DEVELOPMENT AND CHARACTERIZATION OF GELATIN-BASED UNIAXIAL MACRO-POROUS SCAFFOLD BY IONOTROPIC GELATION

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DEPARTMENT OF BIOTECHNOLOGY AND MEDICAL ENGINEERING

C E R T I F I C A T E

This to certify that the thesis entitled "**Development and Characterization of Gelatin-based Uniaxial Macro-porous Scaffold by Ionotropic Gelation"** being submitted by Amartya Amitav for the award of the degree of Bachelor of Technology (Biomedical Engineering) of NIT Rourkela, is a record of bonafide research work carried out by him under my supervision and guidance.

The contents of this thesis, in full or part, have not been submitted to any other university or institution for the award of any degree or diploma.

Place:

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ABSTRACT

For in vitro rearing of long cells such as neuron cells, an artificial tissue engineered construct of appropriate pore structure is of great interest among the modern day researchers. To allow such cells to grow, the mimicking substance must have uniform uniaxial macro-pores. Along with the pore structures, the scaffold must be biocompatible, bioactive, biodegradable etc. To develop and characterize such a construct, a novel approach has been proposed to create a gelatin-based alginate-hydrogel crosslinked with copper ions. The prepared constructs were characterized using SEM, FTIR, pH measurement, swelling study, degradation study and Hemocompatibility study. The copper ions were removed to reduce toxicity. The results showed uniform pores having diameter in the range of 20 to 300 μm and those were visualized like a continuous tubes. Energy Dispersion Spectra (EDS) analysis showed the successful removal of copper ions from the prepared hydrogel. Maximum swelling ratio was found to be 50% and 100% degradation index was observed in all copper-containing samples. Hemocompatibility data showed below 5% hemolysis for all the samples.

Keywords: Gelatin, Alginate, Ionotropic Gelation, Tubular pores

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Grafting (autograft, xenograft etc.) has been a great method to repair and replace damaged or injured body parts by providing cellular level excitation for regeneration and growth of non-damaged cells around the graft area [1]. Autograft is the best method of grafting. But in autograft, the graft is reported to be infection-prone and can activate autoimmune system [19].

Regenerative medicine is a very promising alternative of present day state of grafting [3]. In the current era, it is the most significantly growing branch of engineering. It is an important aspect of tissue engineering in which organ damage is dealt with natural or artificial implantable tissue-alike constructs. The use of tissue engineered constructs was though initially limited to generating replacement of injured skin, in the recent past it has been extended for applications in drug delivery using various biocompatible materials [2]. The tissue regeneration occurs as the body tries to heal itself being supplied with apt types of cells and scaffolds. Without them, an adult body is able only to generate scar tissues [4].

1.1.1. SCAFFOLDS

Scaffolds are supposed to be able for cell adherence, help in division of those cells and activating cell cycles [5]. The significance of scaffold can be fully understood by learning its basic characteristics as follows:

1. Specificity: Scaffolds are supposed only to target the extra cellular matrix around the affected region of the body and to help in the cell regeneration and proliferation till repair of the tissue can be achieved. Tissue constructs can also be used for in vitro studies of cell properties. To achieve specificity, the volume and porosity of the bulk are important parameters of the scaffold.

2. Biocompatibility: Both for in vivo and in vitro applications of scaffold, it is obvious that as they are being exposed to cells, they have to be biologically cell-compatible.
3. Bioactivity: Scaffold are used in drug delivery system. They release cell-activating drugs. Even in tissue replacement, cell activation for adhesion to the ECM and their proliferation is expected. Hence, scaffolds are needed to be biologically active.
4. Scaffolds give mechanical and shape consistency to the tissue imperfection. Unique and different morphological characteristics has been observed for various types of cells while varying the mechanical properties in some of the recent mechano-biological studies [6, 7].

For mimicking different types of tissues in the body, the scaffolds are generally formed either from ceramic materials, hydrogels, nano-metal/composites or their mixtures. For soft tissues, hydrogels have been studied to be the best materials.

1.1.2. HYDROGELS

Polymeric gels swollen with water are known as hydrogel. They consist swell-able crosslinked hydrophilic polymers. To swell in biological environment is their vital property. Hence they can be used in drug delivery and tissue engineering [8-18]. The 3D network structure may be highly structured or the pores are randomly present. It depends upon the crosslinking. It can either be physical or chemical. Hydrogels have high water content. Hence they match natural soft tissue the most. Swelling property of the hydrogels are significant to let the hydrogel have high O_2-, nutrients-permeability [20-25].

1.2. LITERATURE REVIEW

A protein namely gelatin is widely used to make hydrogels due to its swelling-proneness, biocompatibility etc. though mechanical and thermal altercation is required to have construct of regenerative medicinal significance.

1.2.1. GELATIN

1.2.1.1. Source of Extraction

Gelatin is derived from collagen-fiber. Collagen is a water insoluble naturally occurring material. Hence, gelatin is easily available. The gelatin extraction process is done by hydrolysis.

1.2.1.2. Structure and Content

Gelatin is a protein. Amino acid named glycine is present in abundant in gelatin which creates continuous triplet-sequences with either proline or hydroxyproline. Other important amino acids present are alanin, arginin, glutamic acid, aspartic acid etc.

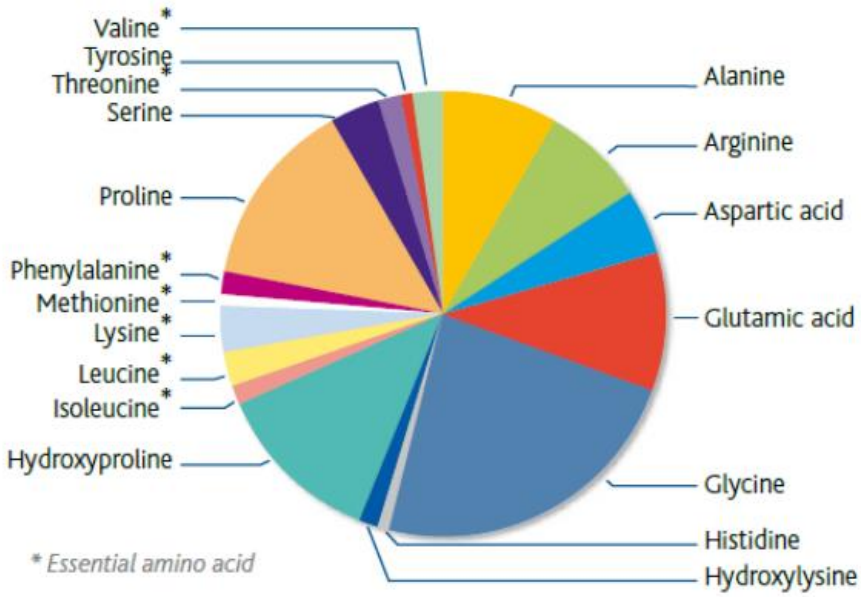


Fig.1.1: Pie chart of distribution of amino acids in gelatin (*Courtesy: PB Gelatins)*

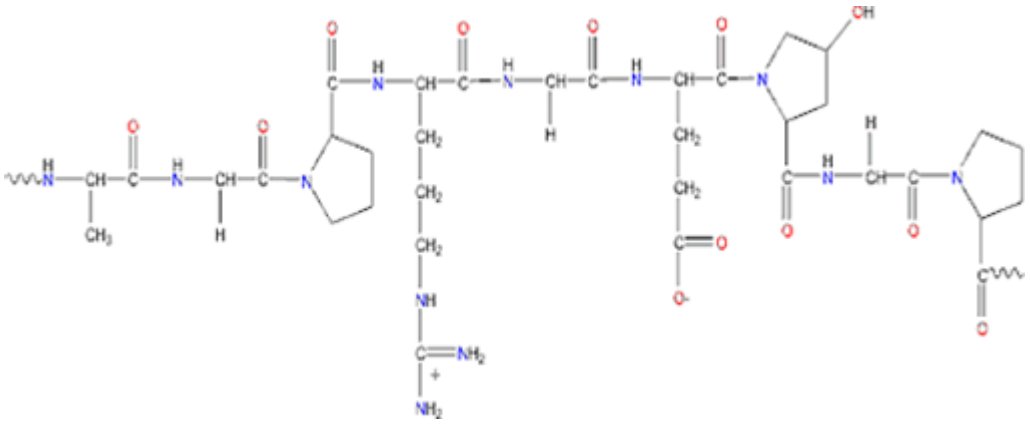


Fig.1.2: Typical structure of Gelatin protein (-Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-)

(Courtesy: niroinc)

1.2.1.3. Characteristic Properties

Gelatin allows nutrients-transfer when used as a scaffold. The hydrogels of gelatin have high swelling property when they are suspended in water-y environment. Gelatin below 35^{o}C turns into a gel which is thermos-reversible in nature. It occurs due to the rearrangement of gelatin's triple-helix structure [26]. This property induces thermal and mechanical instability into the gel [27].

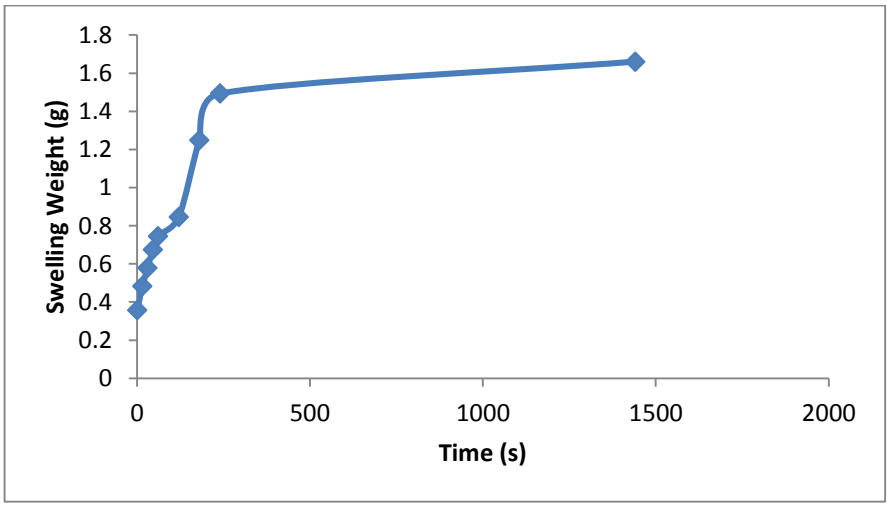


Fig.1.3: Swelling property of gelatin *(Courtesy: Gupta S)*

1.2.1.4. Enhancement in Properties

To make these properties better and exploit all the characteristic properties of gelatin significant for tissue engineering such as porosity, biocompatibility etc., many researchers have proposed cross-linking it with different material or mixing it with stable gel solution. In this proposed novel approach, gelatin solution is mixed with sodium alginate solution cross-linked with Cu^{2+} ions. The hydrogel is prepared to combine the characteristic properties of gelatin and the structural properties of alginate. Furthermore, the hydrogel is made following a certain protocol to create anisotropic macro-pores similar to capillary structures to allow the culture or growth of long cells such as sperm cells, neuron cells etc. Both the pore directionality and diameter contribute to growth of these types of cells.

1.2.2. ALGINTATE

1.2.2.1. Source of Extraction

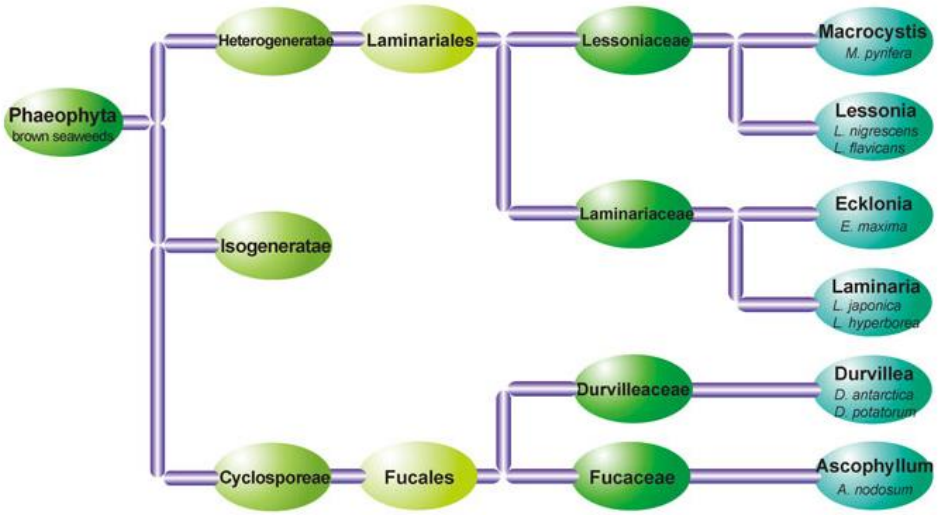


Fig.1.4: Seaweeds suitable for alginate extraction *(Courtesy: kimica-alginate)*

It is generally retrieved from brown algae (Phaeophyceae) [28]. An aqueous alkali solution is used in this process followed by filtering and addition of salt such as NaCl to form sodium alginate salt. This is treated with diluted HCl to form alginic acid. This process gives the commercially available sodium alginate powder which is followed by various purifying protocols [29-30].

1.2.2.2. Structure and Content

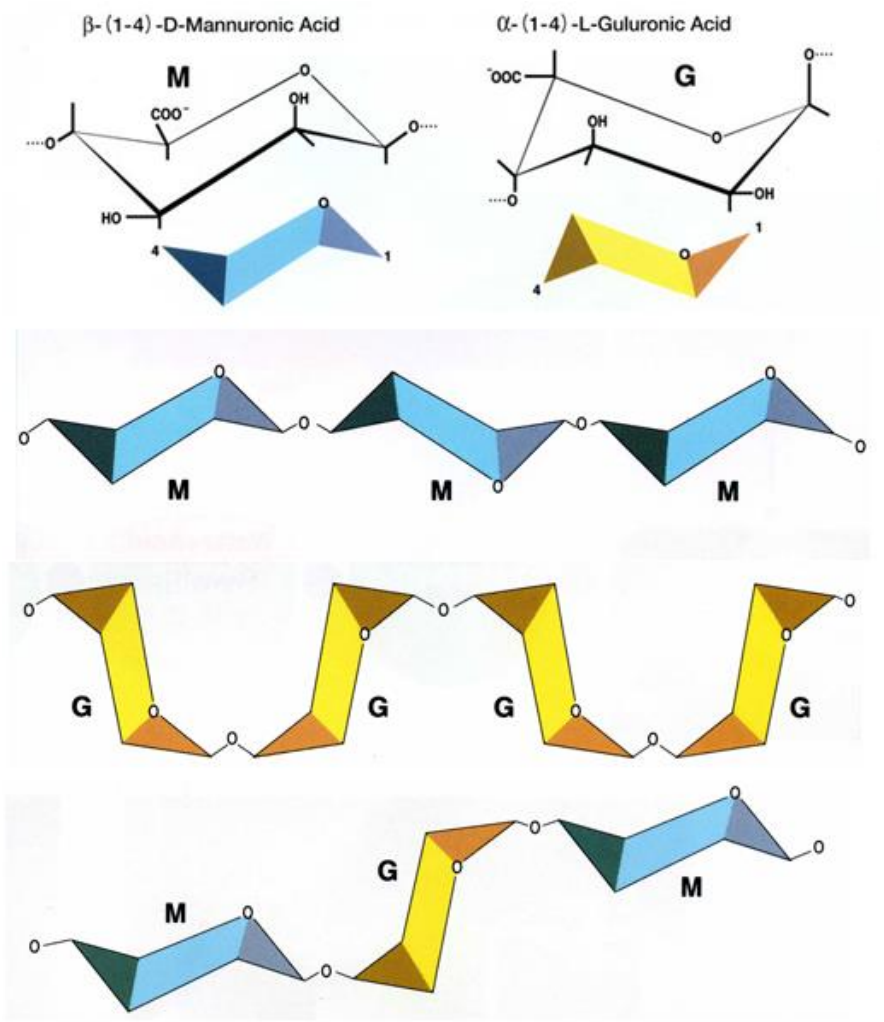


Fig.1.5: Individual blocks of M and G and their three conformational blocks *(Courtesy: kimica-alginate)*

Alginate is a polymer with an excess of negative charge. The formula for alginic acid is $(C_6H_8O_6)n$. It contains linear chain of copolymers consisting blocks formed by combination of β-d-mannuronate (M) and α-l-guluronate (G) residues which are linked by glycosidic bonds. M and G residues are different only by configuration. Hydrogels are formed due to the cross-linking ability of G-blocks with divalent ions such as Cu^{2+}, Ca^{2+} etc. The hydrogel structure is caused by the egg-box motif sandwiching the cations [31].

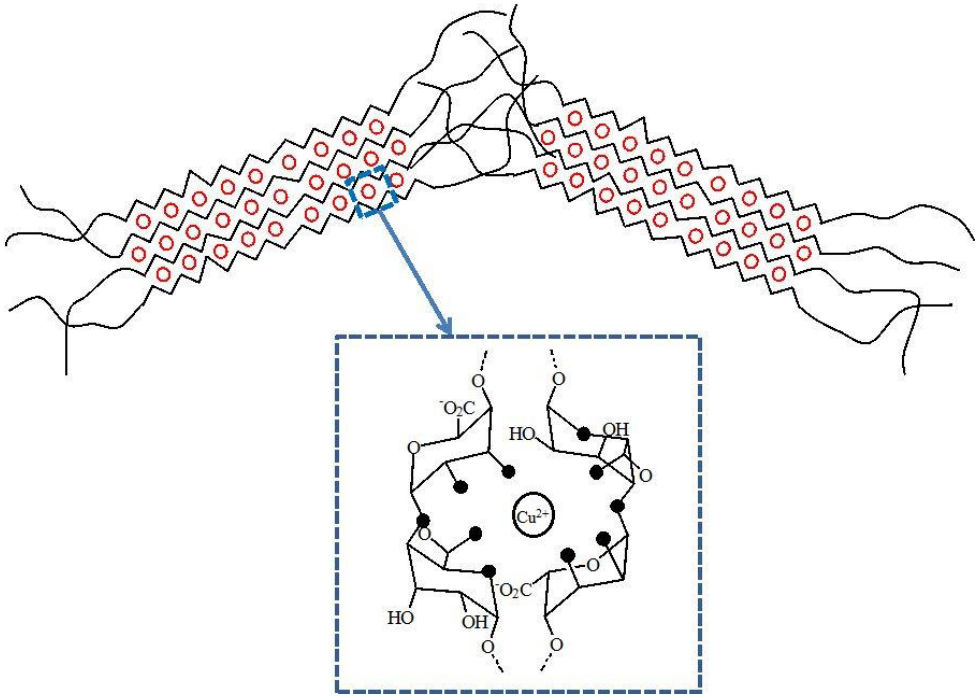


Fig.1.6: Bond of Cu^{2+} ions and $–COO^{-}$ group in the cross-linked alginate hydrogel *(Courtesy: Gulrez et. al* [32]*)*

On an average the molecular weight of sodium alginate sold in the market is 35,000 g/mol. Its viscosity is directly proportional to molecular weight and inversely proportional to pH value. Hence, these are significant control factors of micro-level properties of alginate-based hydrogels [33].

1.2.2.3. Characteristic Properties

Its cross-linking property with divalent ions is widely used in the fields of ceramic industry and material science. But in the field of tissue engineering, the hydrogel formed must have the properties of the specific ECM-mimicking property. According to most of the studied papers, the hydrogels of alginate show very low biocompatibility and immunogenicity [34]. This is a result of presence of naturally-induced impurities in alginate structure. However presence of high amount of G residues may be related to low immune-activity of alginate. Alginate can be used for various tissue engineering aspects if it is proven to be biocompatible.

1.2.3. IONOTROPIC GELATION

Ionotropic gelation is a method in which divalent ions go through a semi-permeable membrane called as primary membrane created on the surface of sol in the initial phase of cross-linking.

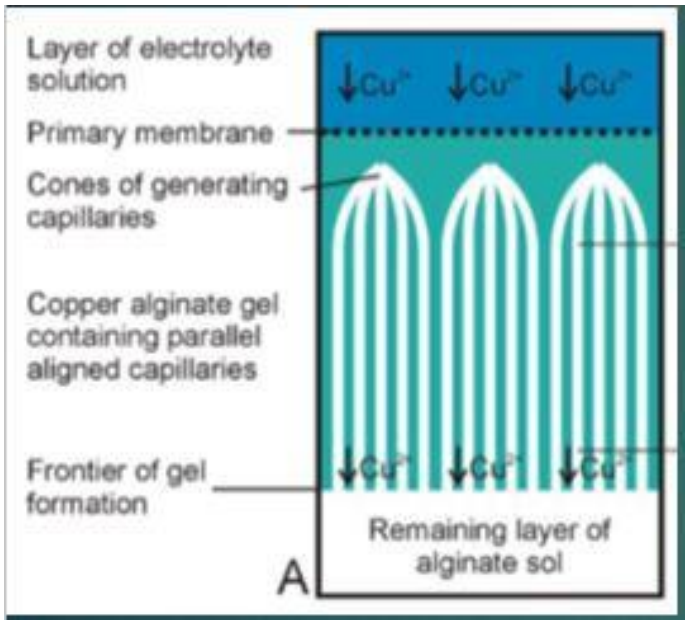


Fig.1.7: An explanatory figure of Ionotropic Gelation (Side view)

The sol is suspended in an electrolyte of divalent ions. If the divalent ions enter the sol uni-axially then they are supposed to diffuse conically and cross-link with the sol creating parallel pores from the same direction. The pore size and pore density are the bulk mechanical property of these kind of hydrogels which are matter of interest. The primary membrane formed in the initial phase has to be manipulated such that the divalent ions can go through parallel pores only into the sol. A mesh-like structure can be used for this. In the proposed ionotropic gelation method, following materials are used and their roles are mentioned.

1.2.3.1. Sol

Alginate is used as the sol in this method. Alginate has negatively charged G blocks which bind with the divalent ion by cross-linking and form the egg-box sandwich motif. Alginate solution is liquid in nature at room temperature until it is cross-linked. This property enables us to use the sol for making parallel-aligned pores. Initially a primary membrane was formed which allows the movement of divalent ions that binds with the G blocks of alginate and form egg-box motif. Alginate is also preferred for its mechanical strength. Its disputable biocompatibility can be compensated by adding biocompatible materials.

1.2.3.2. Electrolyte

copper sulphate solution ($CuSO_4$) is used as the electrolyte in this process. It provides the Cu^{2+} ions to the sol for cross-linking. Cu^{2+} ions are reported to create well-structured uniform pores in the alginate hydrogel when they enter through uniform porous mesh [35]. The uniform gelation may also be contributed to slower gelation rate of $CuSO_4$ than other divalent ions-providing electrolytes.

It is blue in colour and has no odor. It corrodes steel and iron very fast. Hence devices to be used around it must be made up of 304 stainless steel or plastic. Despite of providing good mechanical structure, due to the toxicity of Cu^{2+}, it is biologically toxic to use in regenerative medicines. High percentage of copper sulphate in our body may lead to hemolysis, methemoglobinemia and acute renal failure or kidney injury [36]. The highest amount of $CuSO_4$ that is non-toxic when ingested for our body is around 11 mg/kg. Intravenously, $CuSO_4$ is lethal: 2 mg/kg for guinea pigs and 4 mg/kg for rabbits. Skin contact with it may lead to scratchy skin, allergy etc. *(Source: TOXNET)*. To obtain a biologically active construct using $CuSO_4$ crosslinking, after ionotropic gelatin, the Cu^{2+} must be removed probably by dissolving the Cu^{2+} ions and replace them with non-toxic monovalent ions.

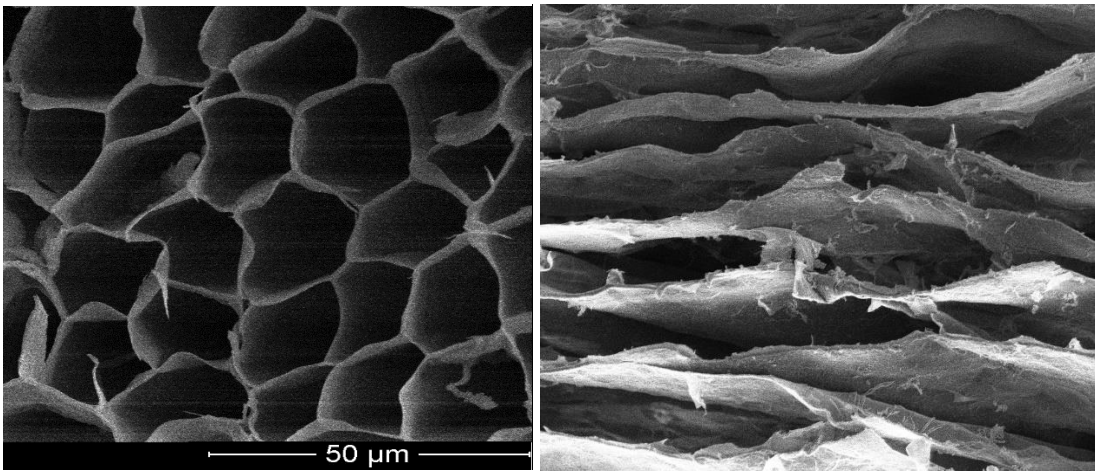


Fig.1.8: Pure alginate cross-linked with copper. Top view and side view. FE SEM 1 200x magnification Nova NanoSEM (*Courtesy: Kasinathan G)*

1.2.3.3. Additive for Biocompatibility

The biocompatibility of alginate hydrogel has to be increased for its usage in our body or for in vitro cell-growth tests. The additive must be non-reactive to other materials being used in the method. **Gelatin** can be one such material. It is highly bioactive and

biocompatible in nature. It has a positive charge residue, so it does get cross-linked with Cu^{2+} ions. Also there is no significant bonds between alginate and gelatin which may negatively affect the suggested method. Gelatin may affect the mechanical stability and uniformity of pure alginate hydrogel solution.

1.2.3.4. Mesh-like Structure

As mentioned before, the uniform parallel pore structure is controlled by the structure of mesh. The mesh can control the pore size and pore distribution. Due to the viscosity of alginate, it does not enter through the mesh structure in upward direction while low viscous solution $CuSO_4$ enters through the mesh easily. A uniform mesh structure is hard to find which is non-reactive with the constituents of the protocol. However, an ingenious structure which is easily available is **KIMWIPE Tissue paper** of KIMTECH science brand can be used as the mesh. It is very fine and smooth in nature. But its microscopic view provides information for its high absorbing qualities. The paper consists of tightly woven fibres which are coarse and lack any extra strands which could have led to tissue paper-debris after usage.

Fig.1.9: Texture of KIMWIPE Tissue paper ***(Courtesy: Stuart Deutsch)***

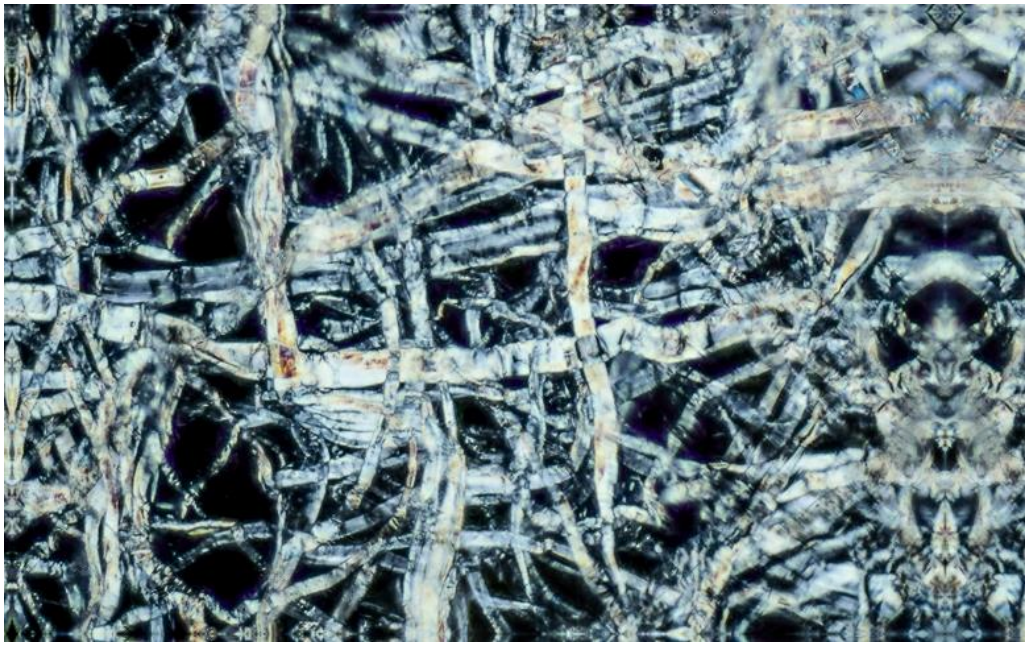


Fig.1.10: KIMWIPE top view. Crossed polars, 40x objective. 1000x total magnification. Four snaps blended with CombineZP macro Soft Stack *(Courtesy: Microbehunter)*

Under Microscope the fibres that make up the tissue are quite coarse and open. It is these spaces between the fibres that give the tissue absorbent qualities. The lack of fine "hairs" on the fibres results in little or no debris left behind after use.

1.2.3.5. Cu^{2+} removal agent

As discussed, the copper content in excess is harmful for the body. So after copper-alginate-gelatin solution to provide mechanically stable and uniform structure, it has to be removed from the construct to get a stable, biologically safe implant or tissue engineered construct. **Nitric acid (HNO_3)** can be used as a copper removal agent. Cu^{2+} ions when exposed to HNO_3, a green colour is found for high copper concentration. After copper gets completely dissolved in the acid, the acid colour becomes blue. The green colour may stick to the hydrogel after copper removal if the copper content is high.

The governing equation is

$$Cu\ (s) + 4HNO_3\ (aq) \rightarrow Cu\ (NO_3)_2\ (aq) + 2NO_2\ (g) + 2H_2O\ (l)$$

The HNO_3 concentration must be monitored to avoid its adverse effects [40].

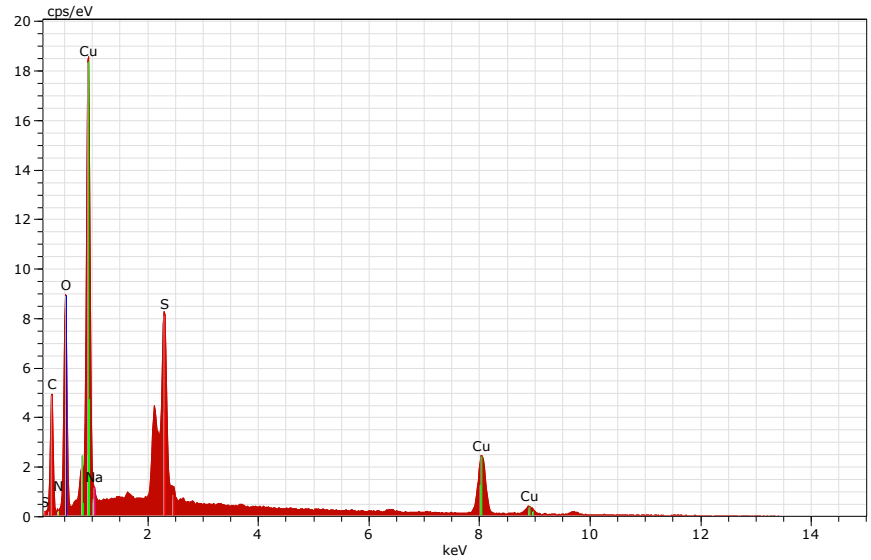


Fig.1.11: EDS graph of pure alginate hydrogel before Cu removal (Cu content =42.5 %)

(Courtesy: Kasinathan G)

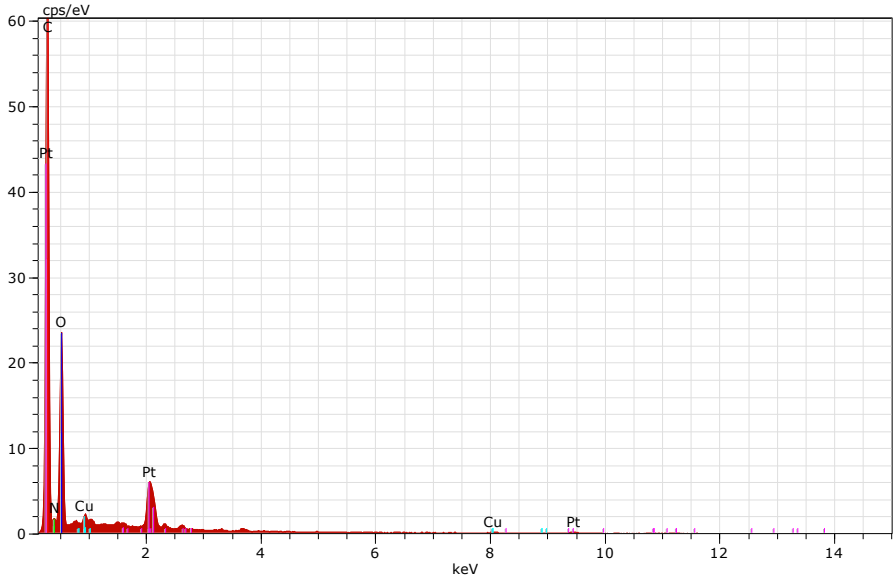


Fig.1.12: EDS graph of pure alginate hydrogel after Cu removal (Cu content =1 %)

(Courtesy: Kasinathan G)

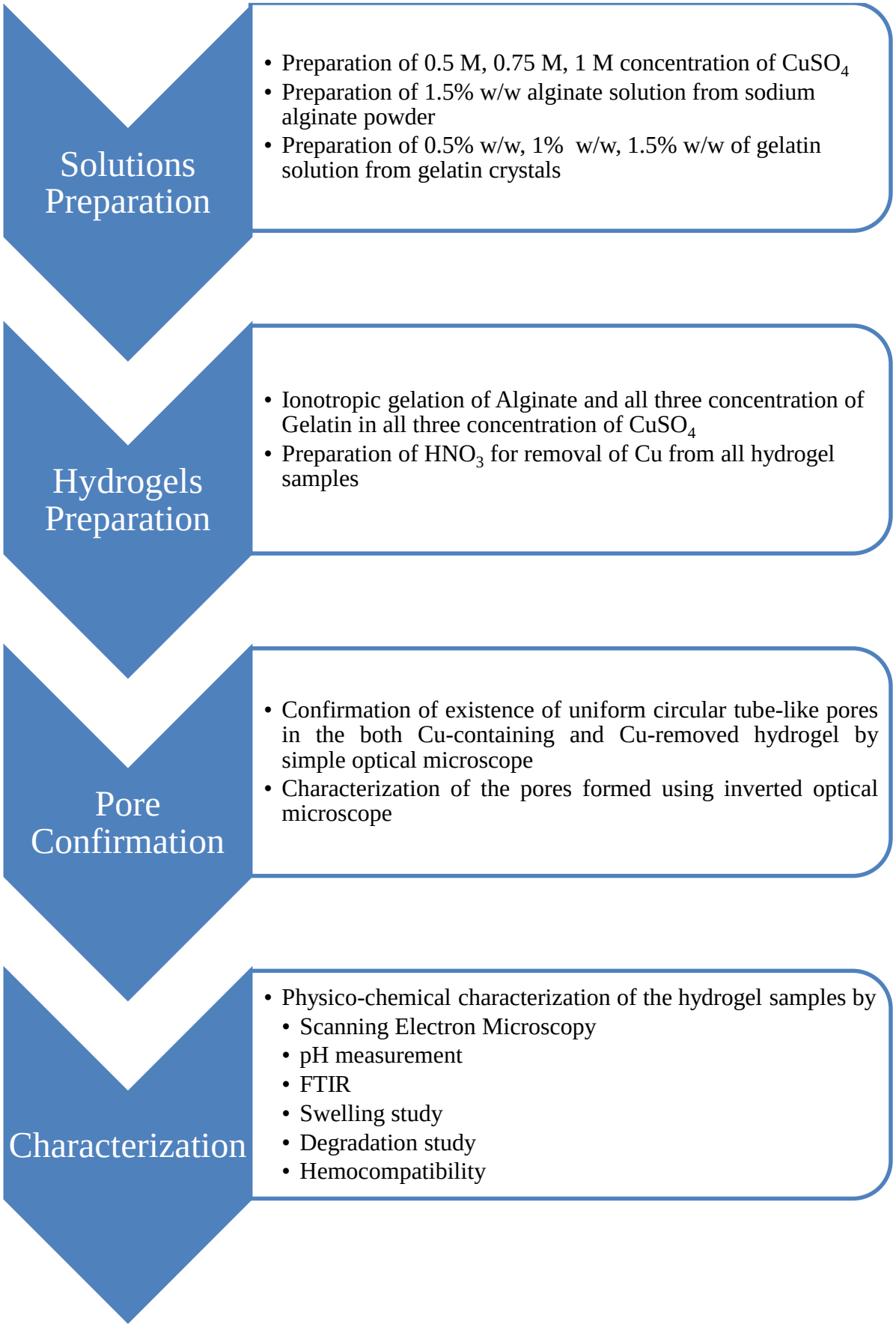
CHAPTER 2

OBJECTIVES AND WORK PLAN

2.1. OBJECTIVES

- ✓ Preparation and optimization of copper alginate-gelatin hydrogel by ionotropic gelation
- ✓ Removal of copper from the alginate-gelatin hydrogel
- ✓ Physical and biological characterization of the hydrogel in presence and absence of Cu

2.2. WORK PLAN


Solutions Preparation
• Preparation of 0.5 M, 0.75 M, 1 M concentration of $CuSO_4$
• Preparation of 1.5% w/w alginate solution from sodium alginate powder
• Preparation of 0.5% w/w, 1% w/w, 1.5% w/w of gelatin solution from gelatin crystals
Hydrogels Preparation
• Ionotropic gelation of Alginate and all three concentration of Gelatin in all three concentration of $CuSO_4$
• Preparation of HNO_3 for removal of Cu from all hydrogel samples
Pore Confirmation
• Confirmation of existence of uniform circular tube-like pores in the both Cu-containing and Cu-removed hydrogel by simple optical microscope
• Characterization of the pores formed using inverted optical microscope
Characterization
• Physico-chemical characterization of the hydrogel samples by
• Scanning Electron Microscopy
• pH measurement
• FTIR
• Swelling study
• Degradation study
• Hemocompatibility

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

Gelatin, Extra pure (GRM019-500G), Sodium Alginate (MB114-100G, CAS No. 9005-38-3), copper (II) sulphate pentahydrate (PCT0104-500G, CAS No. 7758-99-8), Dulbecco's Phosphate Buffered Saline 1X (TL1006-500ML), EDTA disodium salt dihydrate (GRM1195-100G) and Sodium Chloride, A.R. (GRM853-500G, CAS No. 7647-14-5) were bought from HIMDEIA, Mumbai, India. 500ml bottle of Nitric Acid min. 69% w/w GR (61762605001730) was purchased from MERCK, Mumbai, India. 15 ml of blood was donated by an anonymous donor in CWS, Rourkela, India. Kimwipes of KIMTECH science brand was purchased from Axiva Sichem Biotech, Delhi, India.

3.2. METHODS

3.2.1. SOLUTION PREPARATION

3.2.1.1. $CuSO_4$ Preparation

The copper Sulphate solutions of three different concentration were prepared by calculating the crystal weight to be diluted from the desired concentration and volume. 310 ml of $CuSO_4$ solutions of concentration 0.5 M, 0.75 M and 1 M were to be prepared. Thus 77.4 g of $CuSO_4$ was weighed in Mettler Toledo AL204 weighing machine and mixed with distilled water to make 1 M solution of 310ml.

Fig.3.1: Mettler Toledo weighing machine ***(Courtesy: Physiology lab, NIT Rourkela)***

Similarly for 0.75 M solution 58.05 g $CuSO_4$ and for 0.5 M solution 38.7 g of $CuSO_4$ crystals were weighed. The solutions were contained in separate closed glass bottles and were stirred. The stirring was continued for 1 hour and kept for overnight incubation

for homogeneous mixing. To make the other two concentration of $CuSO_4$ homogeneous, slow stirring was again repeated.

The solution after being homogeneous were cautiously stored in a clean and cold place to avoid evaporation and re-crystallization.

3.2.1.2. Alginate Preparation

The alginate solution of 1.5 % w/w concentration was prepared by weighing 1.05 g of sodium alginate powder (white coloured) in Mettler Toledo AL204 weighing machine and dissolving the powder in 70 ml of distilled water. The process of dissolving sodium alginate powder included stirring the alginate powder and distilled water contained in a 250 ml Rivera glass beaker using magnetic bead of length 50 mm at 220 rpm at room temperature for 2 hours on either Tarsons Spinot Digital magnetic stirrer or REMI 5MLH magnetic stirrer (Fig.3.2).

Fig.3.2: REMI 5MLH magnetic stirrer (right) and Tarsons Spinot Digital magnetic stirrer (left) ***(Courtesy: Bioinformatics lab and Physiology lab, NIT Rourkela)***

The solution was further stirred at maximum for 30 minutes to get a completely homogeneous solution.

The solution can be stored for some hours as it doesn't get precipitated or get affected by environmental factors. To avoid air-borne impurities, the beaker was covered with ALUFO Aluminium foil for future applications.

3.2.1.3. Gelatin Preparation

To prepare gelatin solution of 1.5 % w/w concentration, gelatin crystals (light orange-yellow in colour) were weighed for 1.05 g. To dissolve the weighed gelatin powder in distilled water, 70 ml of distilled water contained in a 250 ml Rivera glass beaker was heated to 55 ^{o}C and stirred at 220 rpm on the magnetic stirrer using a 30 mm magnetic bead and Gelatin was added to it. The gelatin was mixed homogeneously in water in 30 minutes. Similarly for 0.75 % w/w and 0.5 % w/w concentration of gelatin 0.70 g and 0.35 g were weighed respectively and added to 70 ml of distilled water separately.

Special care was taken to keep the gelatin above room temperature to avoid its gelling effect. The solution was covered with Aluminium foil until it was required to be mixed with alginate solution.

3.2.1.4. Mixture of Gelatin and Alginate

The prepared gelatin (single concentration) and alginate solution were mixed. Initially the alginate solution was stirred on a magnetic stirrer at 110 rpm and 40 ^{o}C temperature and gelatin was slowly added to prepare 140 ml of sol. After this slow stirring, the rpm was changed to 350 rpm and the stirring continued for 4 hours. Homogeneity was achieved after 4 hours. During the stirring process, temperature was increased at times by 5-10 ^{o}C to avoid clump formation. The mixture is donated as G-SA.

Temperature was maintained greater than 35 ^{o}C and covered with Aluminium foil to avoid contamination and gelation.

N.B: While stirring in magnetic stirrer turbulence was avoided.

3.2.2. HYDROGELS PREPRATION

3.2.2.1. Ionotropic Gelation

For hydrogel preparation, two types of Petri dishes were used namely Cell culture dish (60 mm x 15 mm) of NEST which is made up of plastic or BOROSIL petri dish (75 mm x 15 mm) which is made up of glass. For each G-SA of specific concentration, either two plastic small petri dishes or one single big petri dish was used for incubation in each concentrations of $CuSO_4$.

All the petri dishes (three glass petri dishes or two plastic petri dishes as required) were then coated uniformly with the available G-SA solution thrice with 10 minutes time gap between each of the coatings to let each of the coating dry and create a thin layer on the petri dish surface and walls. The last coating was done with a thicker coating layer. The coating was not completely dried. The significance of coating is to let the solution stick to the surface and to provide ease in taking out the gel.

After 20 minutes, the petri dishes were filled with the G-SA solution till 1 mm space was left at the top of the petri dish. All the petri dishes were covered with Kimwipe tissue paper (half of one piece, i.e., 4.4 in x 4.2 in) such that the Kimwipe was not touching the solution. 0.5 M, 0.75 M, 1 M $CuSO_4$ were poured on the Kimwipe sheets into the petri dishes. To avoid spilling of G-SA solution, initially borders of Kimwipe sheets were wetted with $CuSO_4$. The $CuSO_4$ was then poured on the border of the petri

dishes to let the Kimwipe sheet touch the solution surface and any bubbles in the sols were avoided.

To form primary layer of copper crosslinked alginate on the solution surface, the petri dishes were then poured with $CuSO_4$ through the Kimwipe using a dropper in a uniform manner. After the petri dishes were completely filled with $CuSO_4$, to let the primary layer form, 15 minutes of incubation was allowed.

As the primary layer was formed, the setup was ready for complete ionotropic gelation. The petri dishes were kept in water tanks and completely sunk with available $CuSO_4$ solution of respective concentration. There was a 1.5 mm gap between the topmost part of the petri dish and the topmost layer of $CuSO_4$ solution. The water tanks were covered to avoid impurities, evaporation of $CuSO_4$ solution which may lead to re-crystallization of $CuSO_4$. The tanks were incubated in room temperature for 60 hours.

Fig.3.3: Petri dishes of 0.5G-1.5SA sunk in 0.5 M $CuSO_4$ (right) and incubation period of 0.5G-1.5SA solution (left)

After 60 hours the petri dishes were taken out of the $CuSO_4$ solutions and the Kimwipes were peeled off without disturbing the hydrogel surface. The hydrogels were taken out of the petri dish and submerged in distilled water to retain the gel structure. They were stored in 4^oC for different characterization. The similar procedure was followed for other concentrations of G-SA.

3.2.2.2. Cu Removal

To remove the copper content from the hydrogels, HNO_3 solution was prepared. For complete Cu removal which was observable from the change of colour of the hydrogels from blue to white, three different concentrations of HNO_3 were made and used for Cu removal.

Based on the stock solution of Nitric Acid which is of 15.698 M based on a density of 1.413 g/ml, a formula weight of 63.01 g/mol, and a concentration of 70% w/w, following protocols were followed to make 0.1 M, 0.5 M and 1 M concentration of HNO_3. To make a 1 M solution, slowly 12.741 ml of stock solution was added to 50 ml distilled water. The final volume of solution was adjusted to 200 ml with distilled water. For 0.1 M concentration, 1.2741 ml of stock solution and for 0.5 M concentration, 6.370 ml of stock solution were added to 50 ml of distilled water followed by dilution till 200 ml. HNO_3 concentration controlled the time period of HNO_3-incubation required for complete Cu removal and the mechanical properties of the hydrogels. The Cu-removed samples were also kept in 4 ^oC until further characterization.

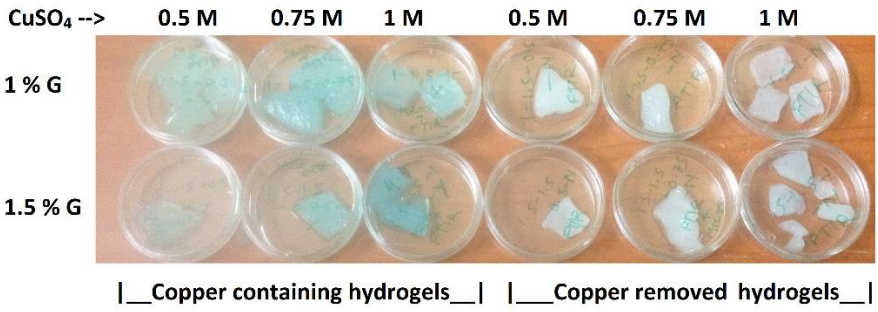


Fig.3.4: Colour change of the hydrogel samples after copper removal

All samples (including HNO_3-treated) were cut in pieces as mentioned in protocols for freeze drying (required for SEM), swelling test, degradation test, hemocompaibility and FTIR.

3.2.3. PORE CONFIRMATION

3.2.3.1. Simple Optical Microscopy

2 cm x 2 cm pieces of the hydrogel samples were taken out of the petri dishes carefully and kept on glass slides. The slides were viewed in simple optical microscope named Olympus iNEA at 5 x and 10 x resolution. The microstructures were observed both from top view and lateral view of cut pieces of the hydrogel.

3.2.3.2. Inverted Optical Microscopy

To get a better resolution image of the hydrogel surface, inverted microscope of OPTIKA was used. For this, the petri dish with wiped bottom surface containing the sample was directly placed on the microscope lens. The microscope was set at 10 x resolution and images were taken with scaling to measure the pore size using ImageJ software.

3.2.4. CHARACTERIZATION

As the pore diameter and density are of utmost importance in this research as the gelatin was supposed to affect the pore structure, scanning electron microscopy was done. To understand the effect of Gelatin, $CuSO_4$ and Cu removal agent HNO_3 on pH, pH measurement was significant. FTIR was done to find the bonds present in the sample. The swelling property and degradation property of the samples were studied to observe how the hydrogels would be affected in vivo environment. Hemocompatibility test was done to find the blood lysis parameter of the samples and hence its biocompatibility.

3.2.4.1. Scanning Electron Microscopy

The gel samples were first cut in pieces of squares of length 20 mm, put in a petri dish and covered with perforated parafilm to provide controlled freeze drying. The samples were kept in freezer at -20 ^{o}C for 2 hours to freeze the water molecules present inside the pores of the hydrogel. Later the samples were taken for freeze drying for 3 days. From the completely dried samples, thin slices were cut and observed under optical microscope to find a suitable pieces for SEM analysis. At last the thin slices having uniform pore structures were gold-sputtered and visualized under SEM. Along with SEM images, EDS (Energy Dispersion Spectrum), a qualitative graph to find presence of copper in the hydrogel, was also plotted.

3.2.4.2. pH Measurement

Calibration of the digital pH meter was done using sample water, pH 7 buffer and pH 10 buffer solution. After this the pH of the gel solution was measured by dipping the probe into the solution. The pH meter equilibrated to the pH value of the sol. To measure the pH of the hydrogels, the probe was pressed upon it.

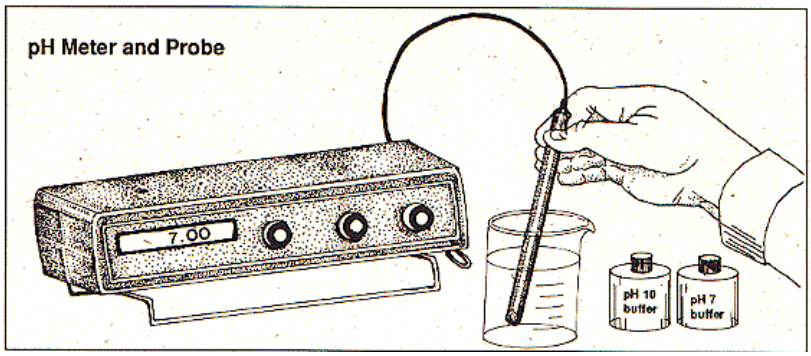


Fig.3.5: pH meter calibration ***(Courtesy:*** Washington State Department of Ecology)

3.2.4.3. Fourier Transform Infrared Spectroscopy

The pieces of each hydrogel samples were cut in size of around 10 mm x 10 mm. The samples were kept in small petri dishes containing little amount of water to avoid drying. The samples were kept in FITR and were pressed by the FTIR sensor and FTIR data was obtained. Before and after each sample processing, the sensor and FTIR surface was cleaned with ethanol. From the obtained data, qualitative FTIR plot for each sample was plotted.

3.2.4.4. Swelling Study

Alginate hydrogels are structurally very stable. Hence they don't swell or degrade easily. To test it, pieces of 10 mm x 10 mm size were cut from the 2 different hydrogel samples and were weighed. Then these pieces were submerged in distilled water and their water binding capacity or swelling property was studied by weighing them at regular interval. Three pieces from each samples were studied for swelling. The characteristic plot was plotted between time and the average swelling ratio.

Swelling ratio (%) = (W_t – W_o) / W_o

Where

W_o – Initial weight of the sample

W_t – Weight of the sample at time t

To increase the swelling property, ion exchange between Cu^{2+} and Na^{+} was studied using the protocol of the proposed swelling method for Ca^{2+}-crosslinked alginate

hydrogel [37]. The ion exchange lead to chain relaxation of Alginate due to repulsion between $–COO^-$ groups.

1 % saline was prepared using 4.0 g NaCl diluted in 400 ml of distilled water. The swelling property was measured as it was done using distilled water for all 18 samples to learn the effect of Cu presence, crosslinking and gelatin. The weighing was stopped when the samples started degrading as saline finally leads to the removal of all the cross-linkers which gave mechanical stability to the gel.

3.2.4.5. Degradation Study

As discussed in the swelling study, saline may lead to initial swelling of hydrogel samples but final degradation is inevitable. To increase the degradation rate, PBS solution was prepared which additionally contains PO^{3-} and KCl than the prepared saline used for swelling studies.. It is used as the solvent. The cut pieces of all 18 samples were weighed in triplets and each sample was suspended in PBS. The degradation property was studied by weighing them at regular intervals until they were completely dissolved or a constant weight was achieved. The characteristic plot was plotted between time and the average degradation index.

Degradation Index (%) = ($W_o – W_t$) / W_o

Where

W_o – Initial weight of the sample

W_t – Weight of the sample at time t

3.2.4.6. Hemocompatibility Test

Hemocompatibility test is done to find if the scaffold initiate RCB lysis or not.

To proceed for Hemocompatibility test, 15 ml human blood was collected with the subject's concern and ethical clearance. It was collected in a falcon tube containing EDTA solution of 1.25 mg per ml of blood to avoid coagulation of blood. 0.9 % NaCl saline was prepared and 18.75 ml of it was added to the blood to maintain 4:5 ratio [38]. Each sample were cut in 5 mm x 5 mm size and were kept in separate falcon tubes. In those falcon tubes 0.5 ml of diluted blood and normal saline to make up the final volume to 10 ml were poured. Positive control was prepared by dilution of 0.5 ml of 0.01 N HCl to 10 ml in the falcon tube without the presence of sample. For negative control 0.5 ml of normal saline was used. The falcon tubes were kept in OSWORLD B.O.D. Incubator (JRIC-10) at room temperature for 60 minutes followed by centrifugation at 4000 rpm for 10 minutes. The optical densities of the supernatant of the samples were measured at 545 nm using UV-Visible spectrophotometer [39].

The calculation of hemolysis (%) is as follows:

$$\text{Hemolysis (\%)} = 100*(O.D._{x} - O.D._{-ve}) / (O.D._{+ve} - O.D._{-ve})$$

Where

$O.D._{x}$ – Optical Density of supernatant collected from the sample tube

$O.D._{-ve}$ - Optical Density of supernatant collected from the negative control tube

$O.D._{+ve}$ - Optical Density of supernatant collected from the positive control tube

CHAPTER 4

RESULTS AND DISCUSSION

4.1. PHYSICAL CHARACTERIZATION OF SA-G HYDROGELS

To have ease in sampling the samples are called from now on as in following format:

Concentration of Gelatin-Concentration of Alginate-Concentration of $CuSO_4$-Cu (y/n)

For example, a hydrogel having gelatin of 0.5 % w/w, alginate of 1.5 % w/w and $CuSO_4$ of 1 M concentration with Cu removed is written as 0.5-1.5-1-n. In some of the characterization the following numbering has been used.

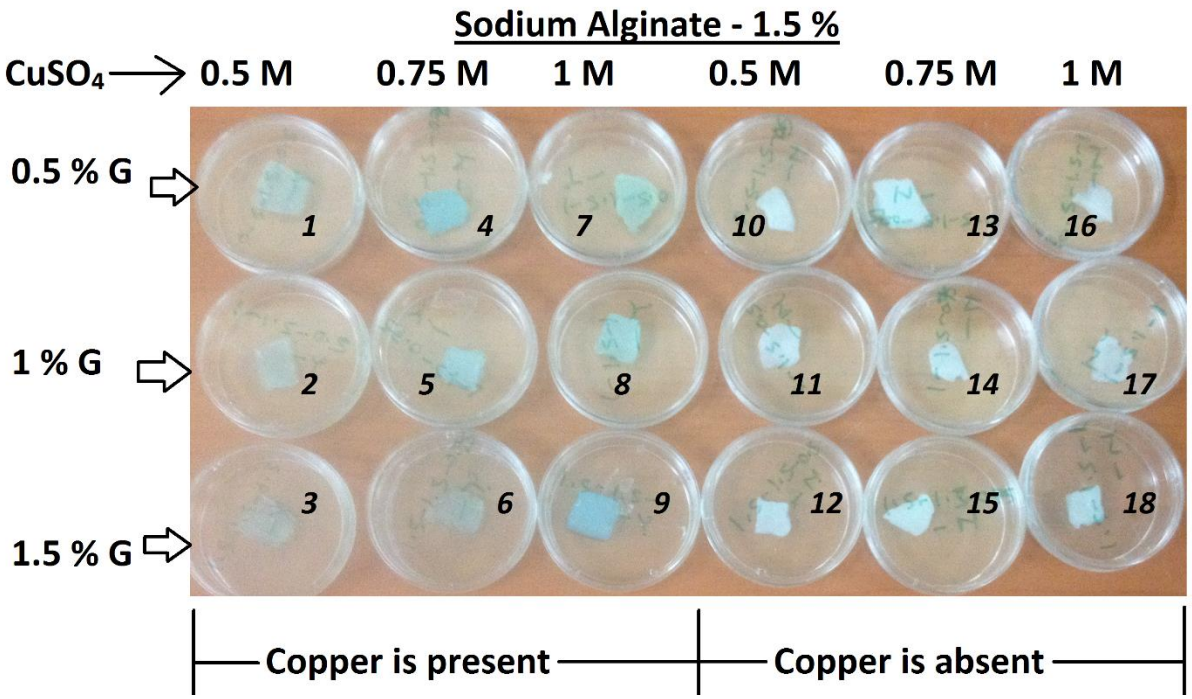


Fig.4.1: All samples in small petri dishes. Numbering has been done for convenience in characterization

As we can observe, the Cu-present samples were blue in colour and Cu-removed samples were white in colour. The blue colour increases with concentration of $CuSO_4$ which depicts the increased amount of crosslinking. Increase in gelatin percentage increased the softness of the sample but the samples had less breakability. Higher concentration of $CuSO_4$ also increased the gel's mechanical strength due to more no. of crosslinking.

0.5-1.5-1-y 0.5-1.5-0.5-y

Fig.4.2: Significant colour difference between sample 7 and 1 due to difference in Cu content

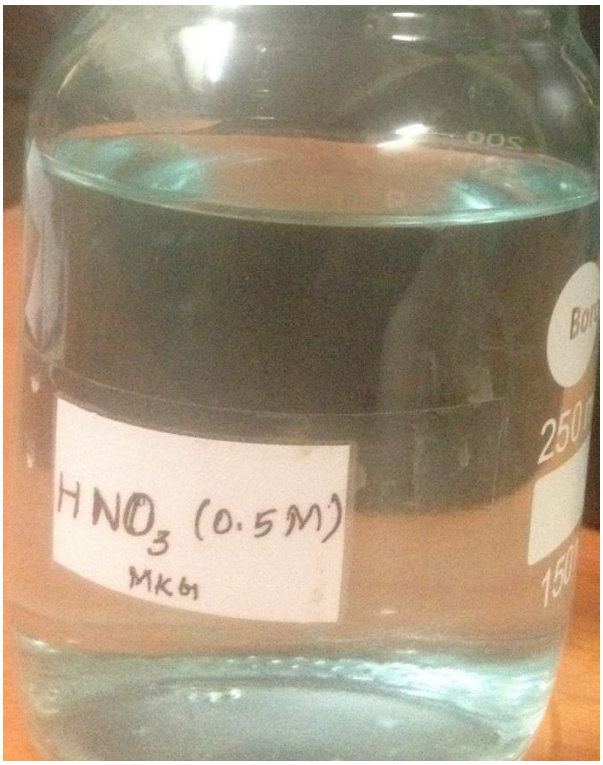


Fig.4.3: The colour of transparent HNO_3 solution after Cu removal

When Cu was removed, the samples shrunk in volume. It occurred due to the dissociation of some of the egg-box motifs and Cu loss. The removal procedure took more time for samples having more Cu concentration. Similarly, the samples were less affected by HNO_3 environment structurally for higher values of both Gelatin and Cu. It might be due to the

balance between less contributions of egg-box motifs in forming the gel structure and presence of more no. of Cu ions in excess.

Keeping the Cu-present samples in distilled water removed the excess of $CuSO_4$. Hence this can be used as the primary Cu removal process. It is based on non-swelling property of crosslinked alginate gels and it works better in gels having less concentration of gelatin.

4.2. OPTICAL MICROSCOPY

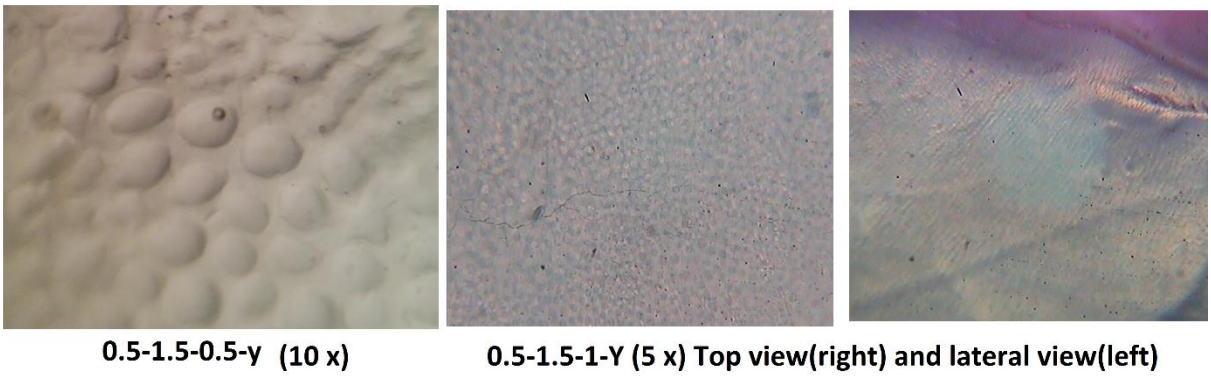


Fig.4.4: Simple optical microscopy of sample 1 and 7

As we can clearly observe from the above simple light microscopic images, the uniform pores are visible from top view. In the third figure, the lateral view of sample 7, the capillary structures are visible at ~45^o angle. Another inference from these microscopic images was obtained that the pores have different diameter. The pore density and pore distribution of the samples were observed qualitatively. More amount Cu ions helps in increasing the pore density.

Using inverted microscopy, samples were visualized under normal light and under green fluorescent light for better view. It can be observed that the size of pores have been reduced for Sample 16 w.r.t Sample 7, 12 w.r.t 3, 18 w.r.t 9 and 17 w.r.t 8. These samples are related in terms of presence of Cu. The Cu removal affects the pore size drastically.

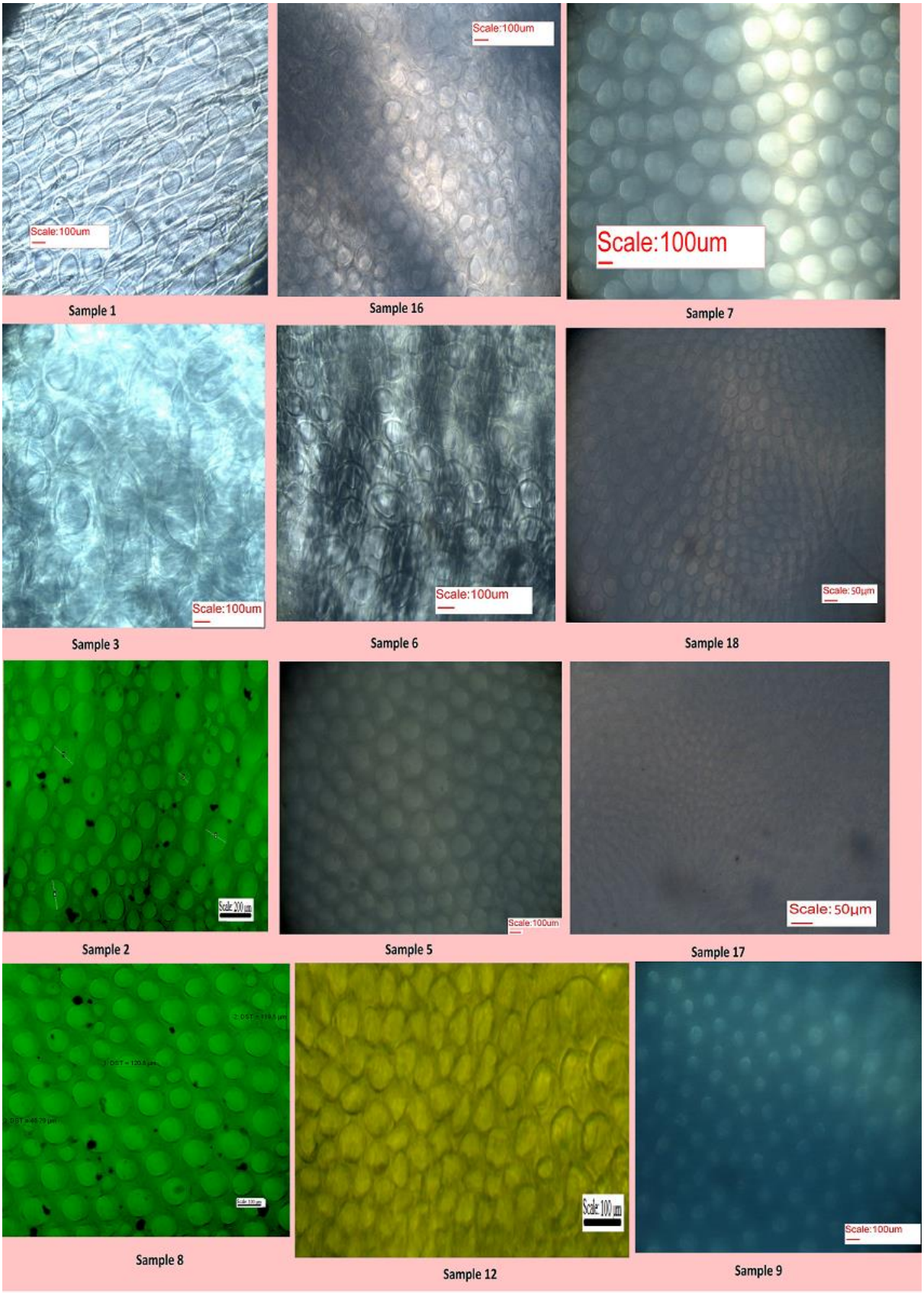


Fig.4.5: Inverted microscopy top view of various samples

Sample No.	Pore Size (µm)	Approximate Pore Density (mm^{-2})
1 (0.5- 1.5- 0.5- y)	164.4±70.5	26
2 (1- 1.5- 0.5- y)	158.1±81.3	55
3 (1.5- 1.5- 0.5- y)	239.9±59.2	8
5 (1- 1.5- 0.75- y)	149.0±54.2	20
6 (1.5- 1.5- 0.75- y)	131.3±51.3	32
7 (0.5- 1.5- 1- y)	190.1±82.0	19
8 (1- 1.5- 1-y)	117.3±71.3	48
9 (1.5- 1.5- 1- y)	130.6±79.9	26
12 (1.5- 1.5- 0.5- n)	73.8±52.9	50
16 (0.5- 1.5- 1- n)	127.1±48.0	43
17 (1- 1.5- 1-n)	65.5±55.5	65
18 (1.5- 1.5- 1- n)	78.0±44.4	52

Table.4.1: Comparison of average pore size and pore density (approximate value)

4.3. SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy gave a high contrast view of the pore structures present in the hydrogels. It was learnt that freeze drying affect the pore morphology and reduces its size. The pores got cracked. The unbound gelatin strands were also observed which might reduce the cells transportation rate in the hydrogel.

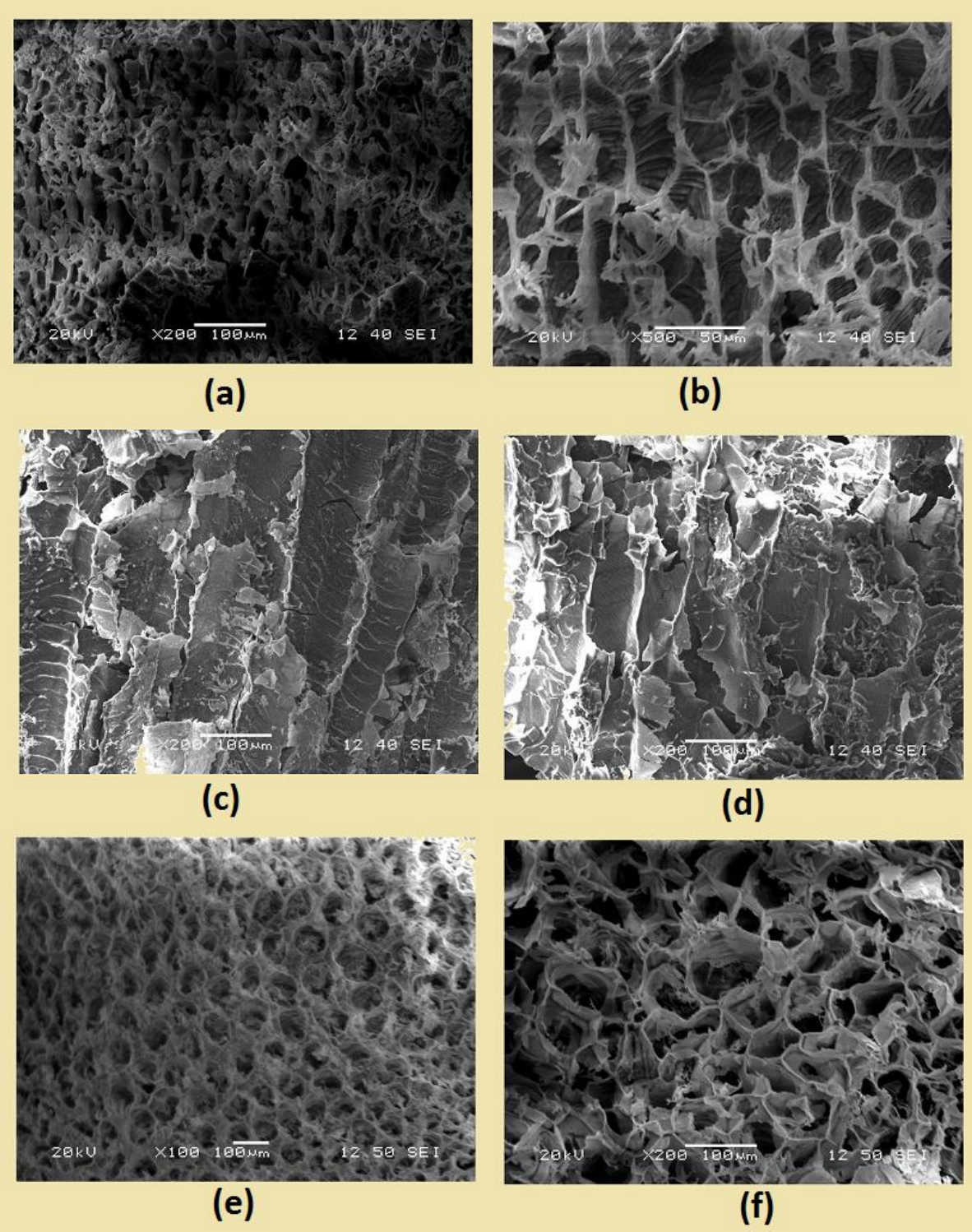


Fig.4.6: SEM of (a) top view of sample 1 at 200x, (b) top view of sample 1 at 500x, (c) and (d) lateral view of sample 4 at 200x, (e) sample 7 at 100x, (f) sample 7 at 200x

The pore diameter of sample 4 was found out from the lateral view as 100.6 µm. We could also infer that for Cu concentration, sample 7 is well-structured than sample 1.

Besides SEM images, EDS graphs were also plotted to show the Cu % present in the hydrogel.

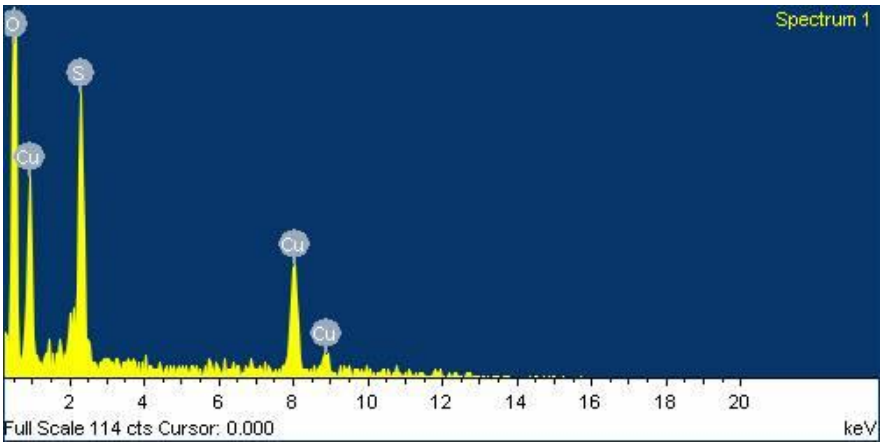


Fig.4.8: EDS of sample 4 with copper (0.5G-1.5SA-0.75 $CuSO_4$) (Cu content is 29.92 %)

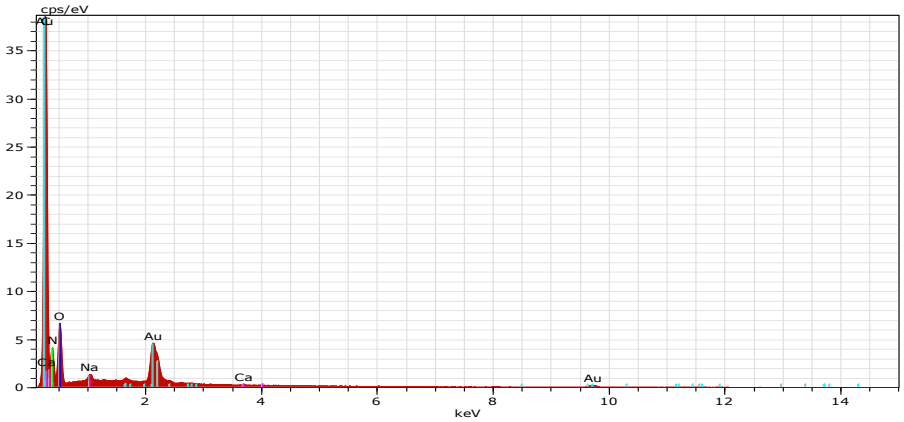


Fig.4.7: EDS of sample 4 after copper removal (0.5G-1.5SA-0.75 $CuSo_4$) (Cu content is 0%)

From these EDS curves, it is clearly visible that the Cu content present in both the samples are high.

4.4. pH MEASUREMENT

The pH value of a scaffold is very important. The scaffold to be implanted in our body must match to the body pH. Thus, though this work is mainly based on the mechanical structure, the sample sol's pH and the pH of the hydrogels were obtained. For future work, this data can be referred while manipulating the pH values.

The pH value of 0.5G-1.5SA solution mixture was found to be 6.5.

Sample	**pH value**
1.5-1.5-0.5-y	5.3
1.5-1.5-0.5-n	5.1 (after HNO_3 dilution)
0.5-1.5-0.5-y	5.2
0.5-1.5-0.5-n	5.3 (after HNO_3 dilution)
1.5-1.5-1-y	5.5 (after Cu dilution)
1.5-1.5-1-n	5.2 (after HNO_3 dilution)
0.5-1.5-1-y	5.5
0.5-1.5-1-n	5.3

Table.4.2: pH values of samples having optimum concentration of gelatin or $CuSO_4$ or Cu presence

It was observed that the excess of copper ions present on the hydrogel was the reason of acidic nature of Cu-present gels. Again for Cu-absent gels, excess of HNO_3 played a major role in increasing the acidic nature of the samples. Hence the excess of Cu ions and HNO_3 were removed and pH was measured.

4.5. FOURIER TRANSFORM INFRARED SPECTROSCOPY

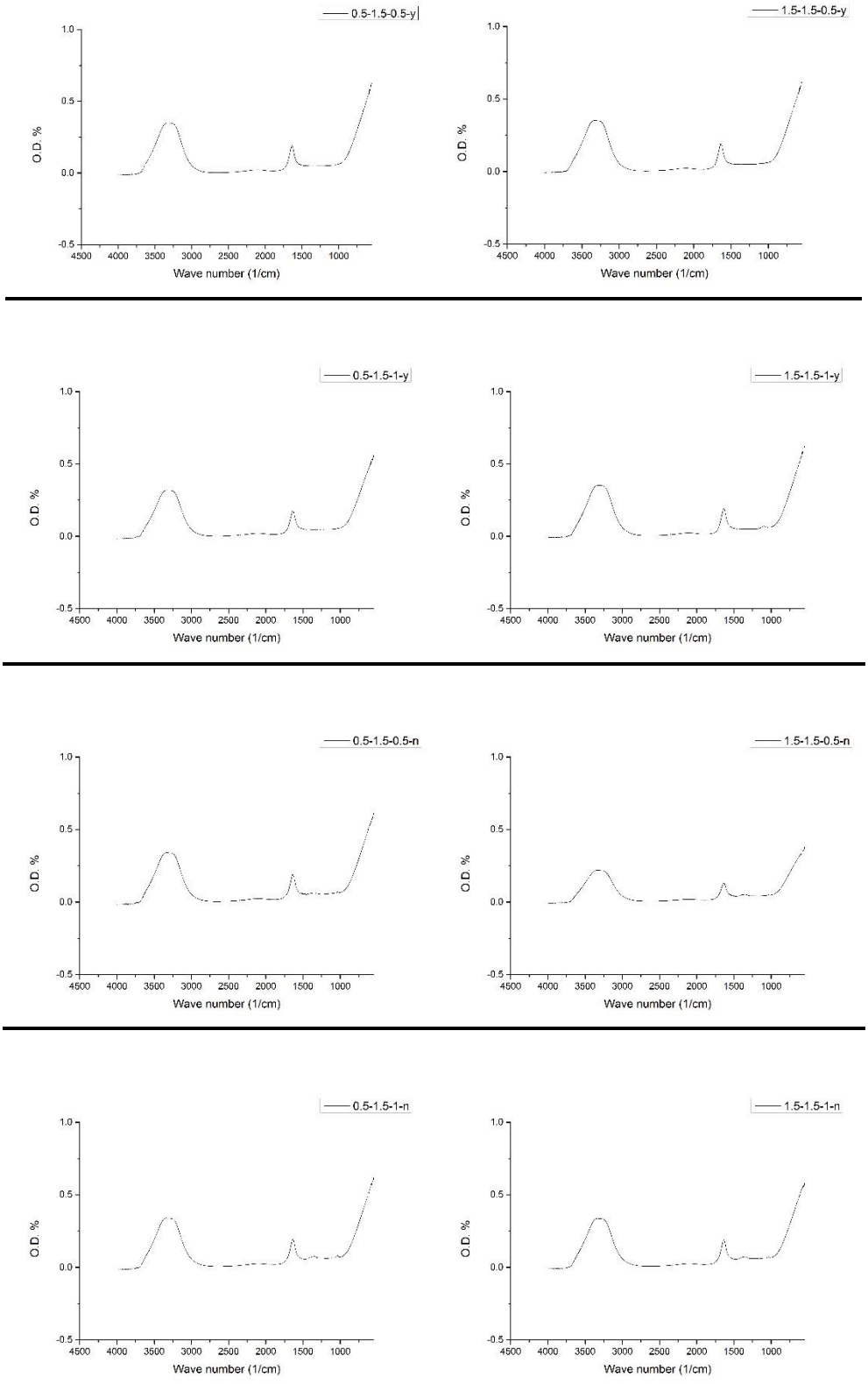


Fig.4.9: FTIR data of the samples with peaks at wave no. ~3500 mm^{-1} and ~1700 mm^{-1}

The first peak at ~3500 cm^{-1} wave no. was related to OH^- stretching and the second peak at ~1700 cm^{-1} was related to O-C-O^- stretching. For lower concentration of copper in copper-present hydrogels there is no effect of gelatin on the bonds. Similar observations were found for higher concentration of copper removed from copper-absent hydrogels. In rest of the two cases, gelatin affects the O.D % values of the peaks. For a constant value of gelatin in Cu-present gels, smaller peaks were observed for higher concentration of $CuSO_4$. Smaller peaks were also observed when lower concentration of $CuSO_4$ was used and Cu was removed.

4.6. SWELLING STUDY

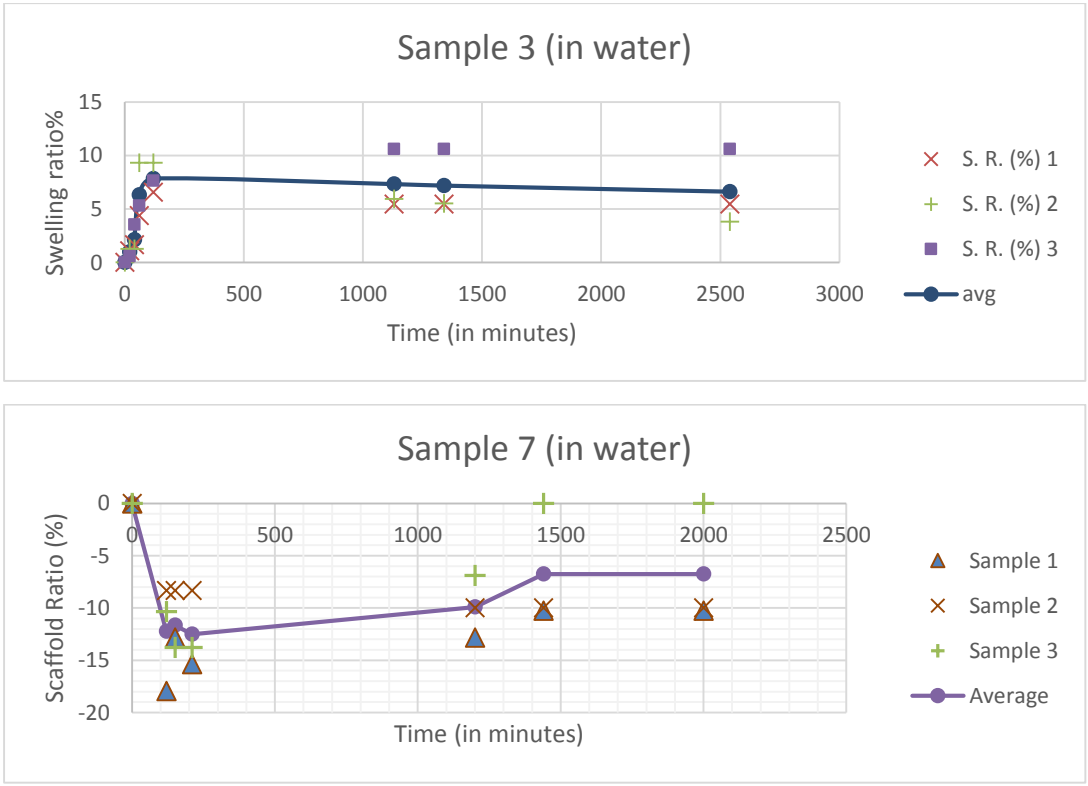
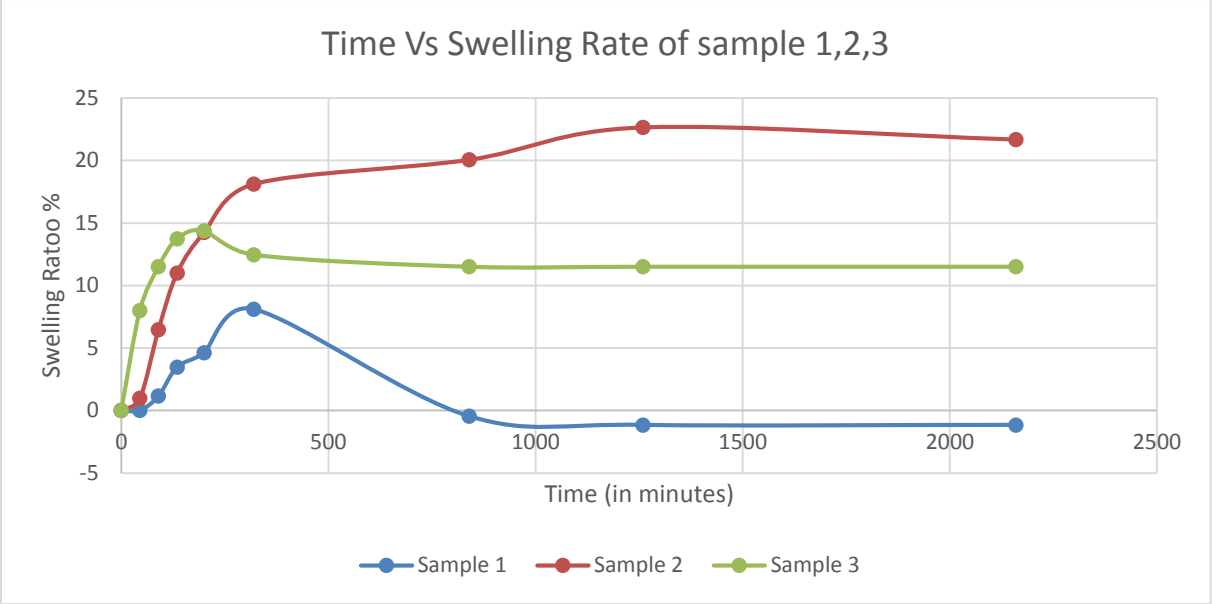


Fig.4.10: Swelling property of sample 3 and 7 in distilled water

As we can see, the hydrogels were not at all swelling in presence of distilled water. The sample 7 showed degradation whereas in sample 3, presence of gelatin lead to a 7 % of swelling ratio. Hence swelling study for all samples was done using 1% NaCl.



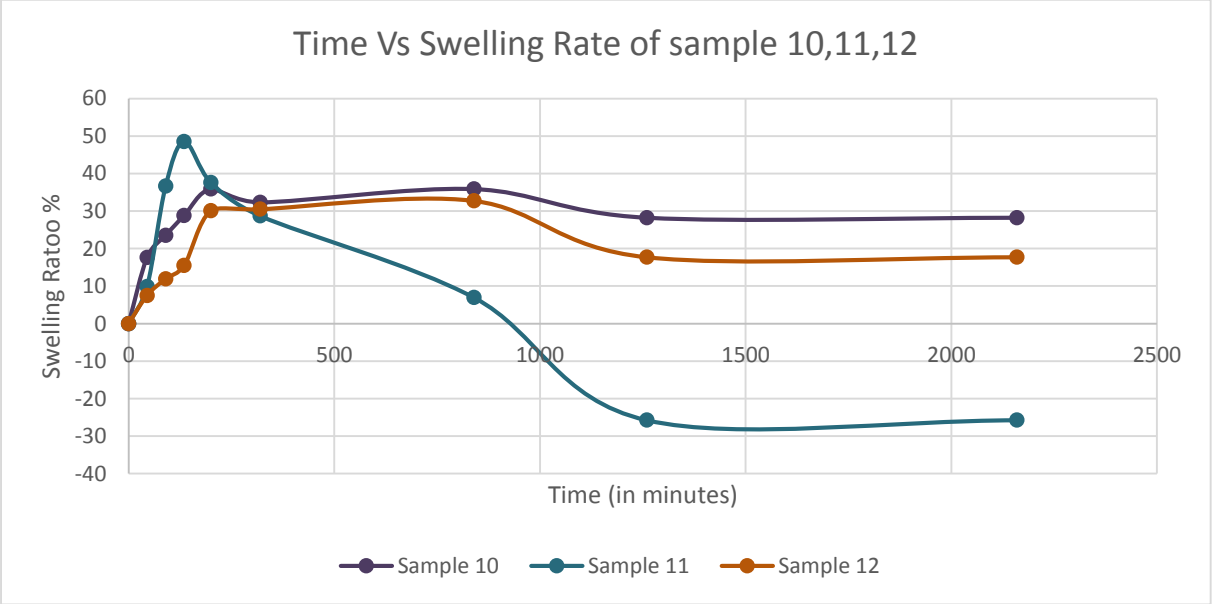


Fig.4.11: Swelling property for increasing gelatin concentration with 0.5 M $CuSO_4$ concentration in Cu-presence and Cu-absence

In presence of copper, medium amount gelatin led to higher swelling (~23 %). In absence of copper, the same hydrogel swelled the most (~50%) but it degraded very fast. Similarly at low gelatin concentration, Cu-present hydrogel degraded very fast but when Cu was removed, a constant swelling ratio of ~21% was observed for both 0.5 % G and 1.5 % G. It implies that 1

% G samples swell the most but have very low mechanical stability. Better swelling rate was observed for other concentration of G when Cu was removed.

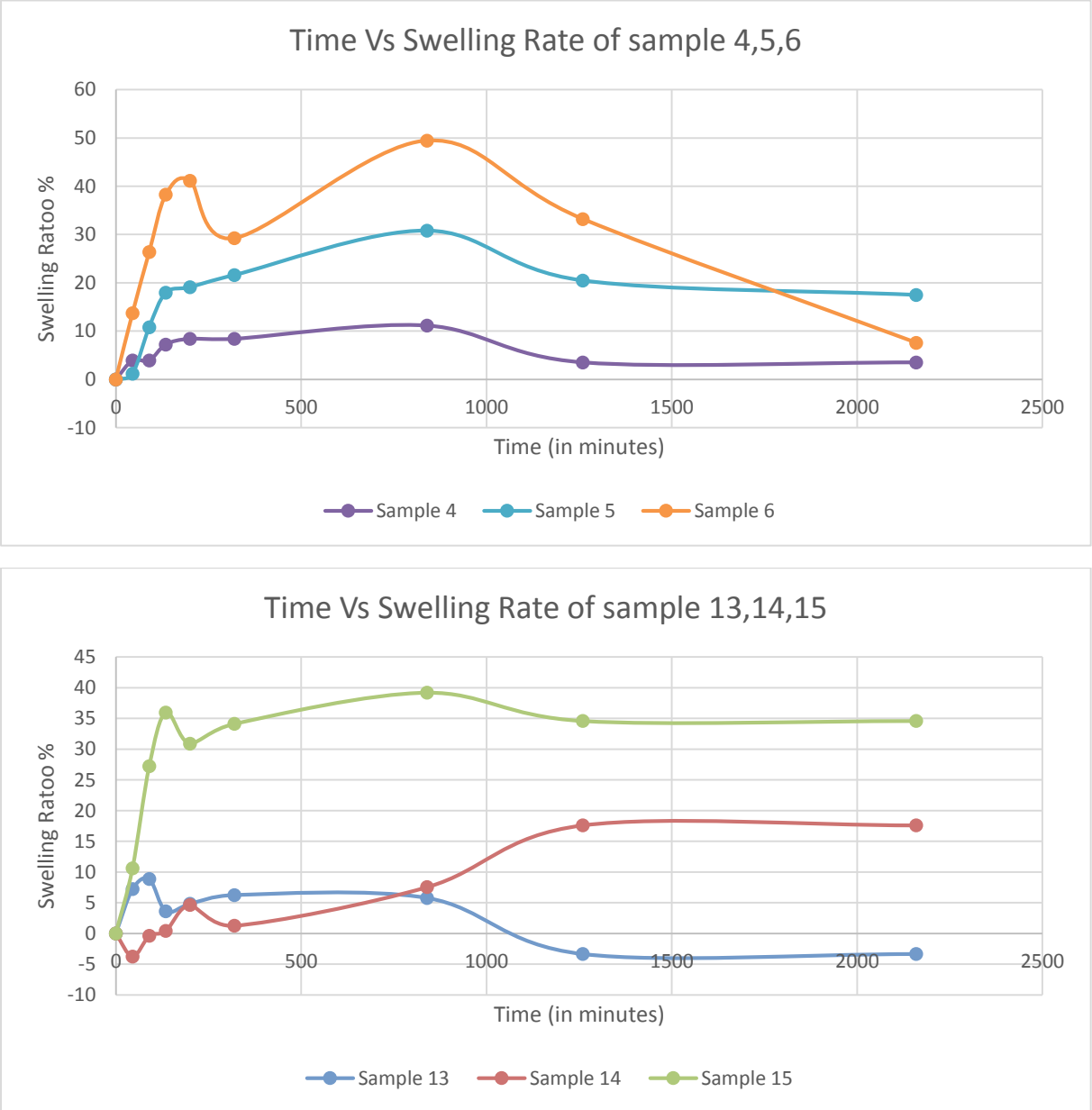


Fig.4.12: Swelling property for increasing gelatin concentration with 0.75 M $CuSO_4$ concentration in Cu-presence and Cu-absence

As the concentration of $CuSO_4$ was increased, the swelling property of the hydrogels became dependent in presence of gelatin. Lower gelatin concentration led to very low swelling ratio. Similarly, around 50% swelling ratio was observed for highest conc. of G when Cu was present. Cu removal led to reduction in swelling ratio.

The highest swelled hydrogel was degraded after 10 hours in a very fast rate.

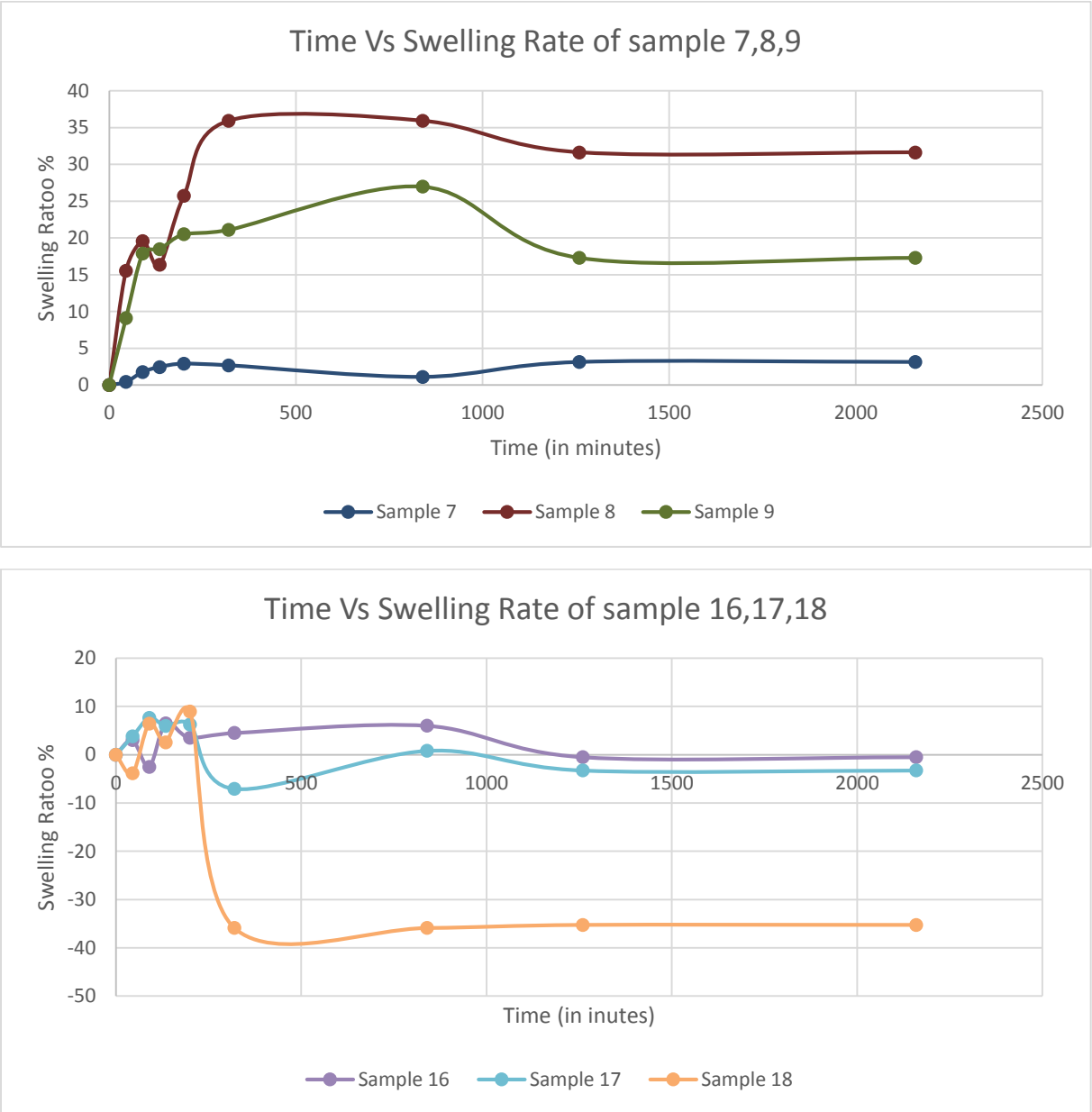


Fig.4.13: Swelling property for increasing gelatin concentration with 1 M $CuSO_4$ concentration in Cu-presence and Cu-absence

Removal of Cu did not initiate swelling at all. The samples degraded from the start having a maximum swelling ratio of 10 % only. In presence of copper, 1 % G hydrogel showed around 35 % of swelling ratio similar to the sample 2.

4.7. DEGRADATION STUDY

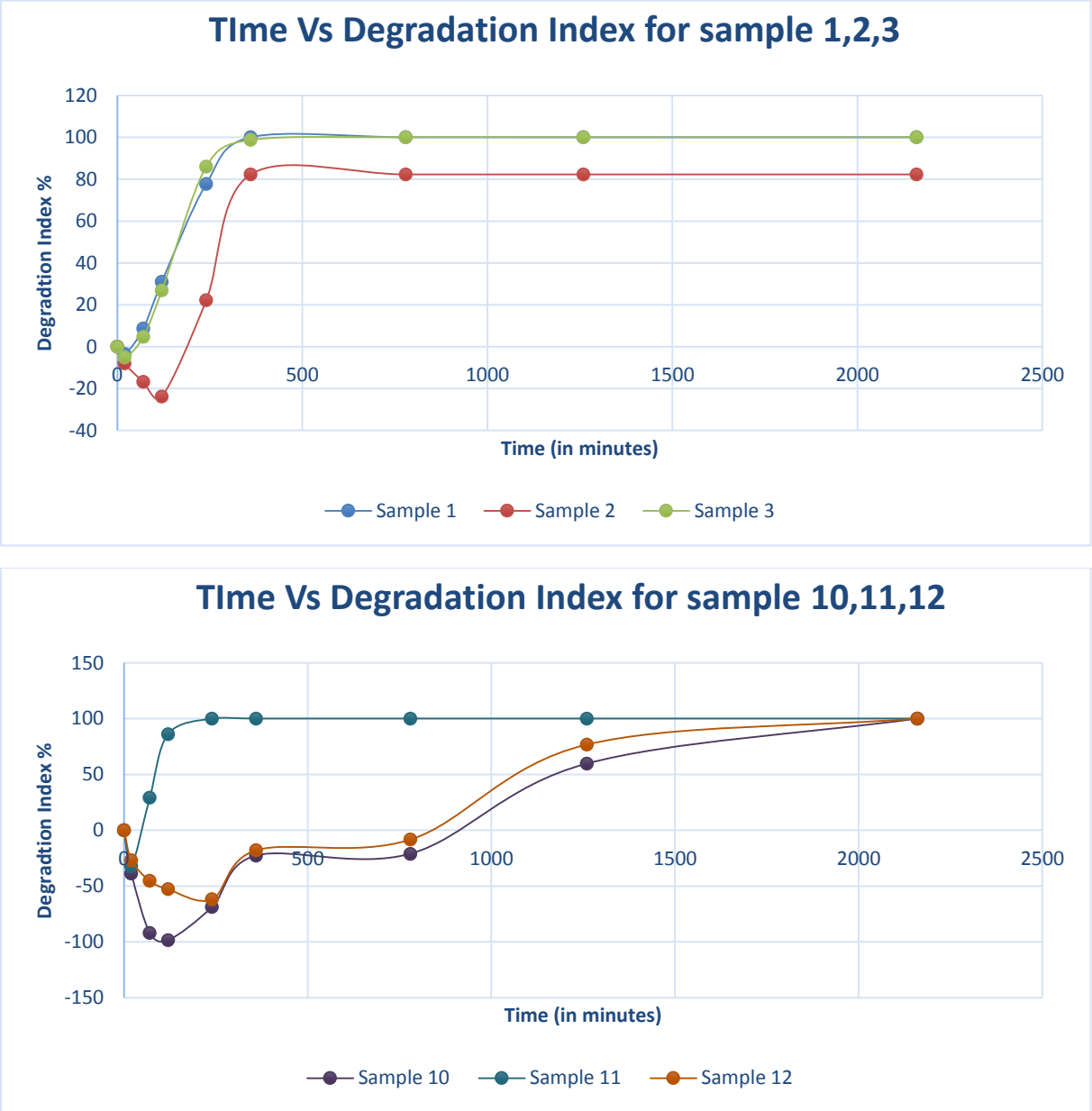


Fig.4.14: Degradation property for increasing gelatin concentration with 0.5 M $CuSO_4$ concentration in Cu-presence and Cu-absence

Very fast degradation was observed in all the samples when Cu was present. It might be due to dissolution of egg-box motif by the PBS. But samples when Cu was removed were found to swelling initially before started degrading after ~12 hours.

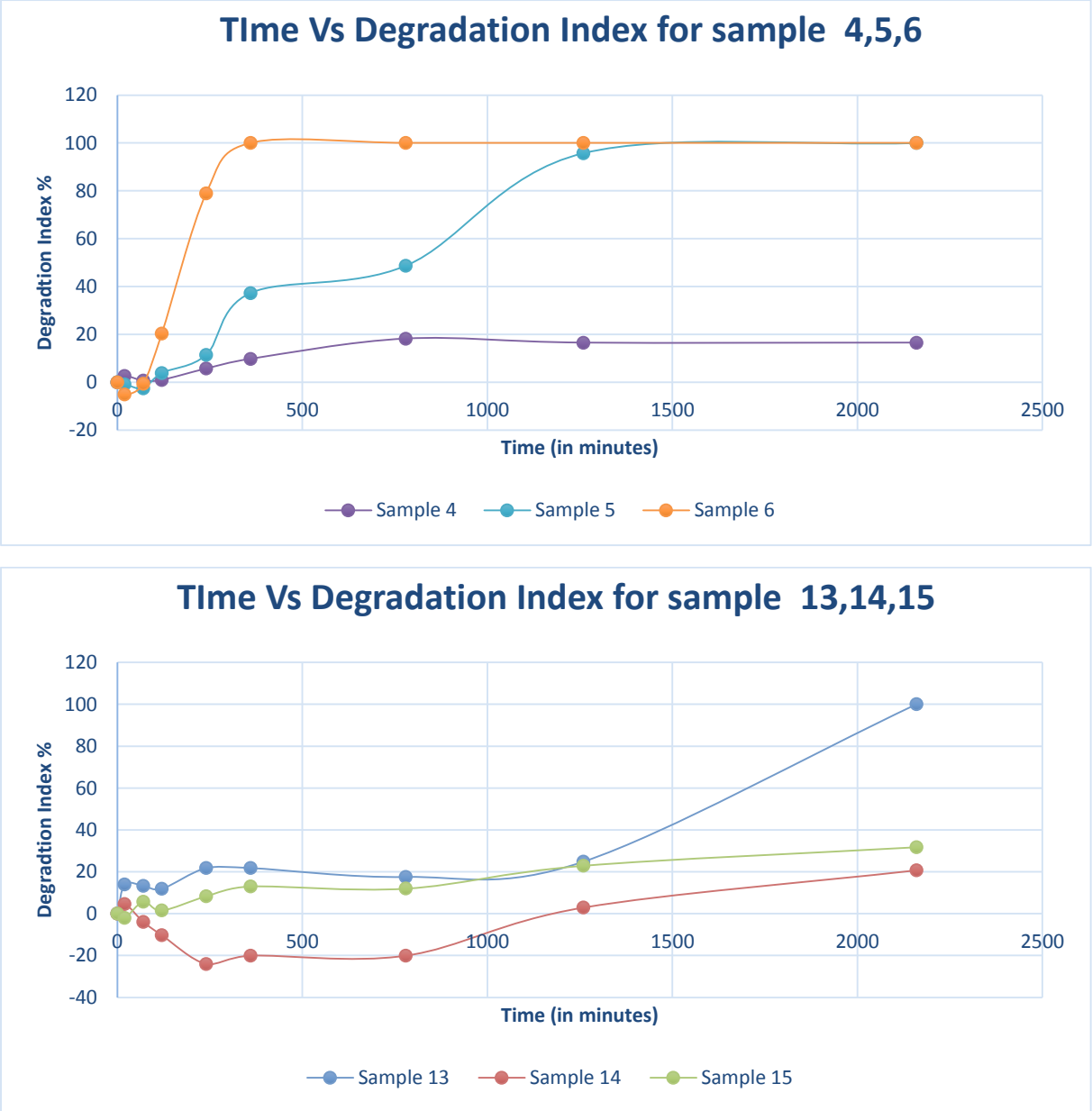


Fig.4.15: Degradation property for increasing gelatin concentration with 0.75 M $CuSO_4$ concentration in Cu-presence and Cu-absence

For medium concentration of $CuSO_4$, the samples had degradation rate proportional to the gelatin concentration in presence of copper. A completely opposite trend was observed in

absence of copper where lower concentration of gelatin degraded in a faster rate. Other concentrations of gelatin were swelling-prone at initial stage and degraded very slowly later.

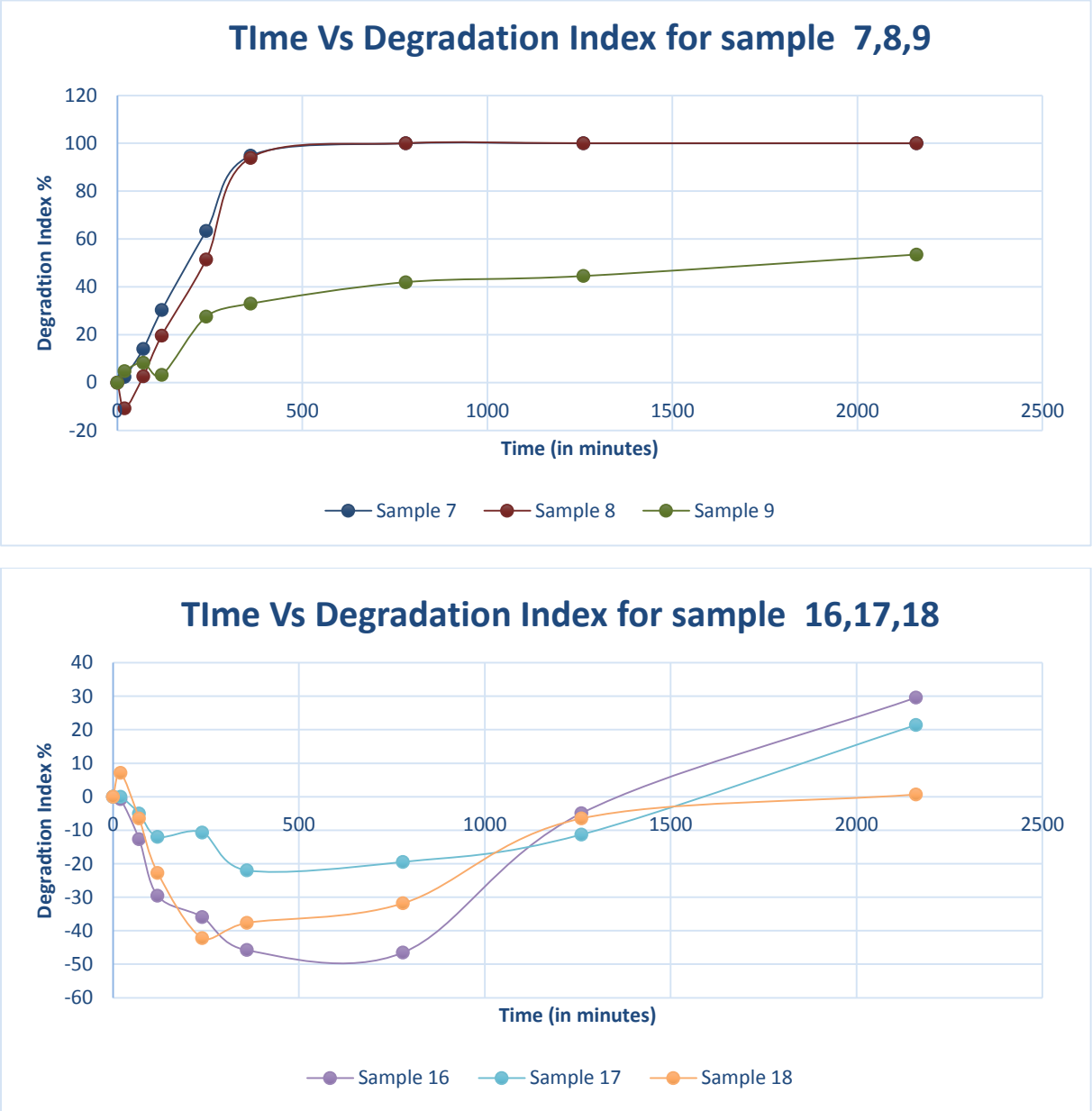


Fig.4.16: Degradation property for increasing gelatin concentration with 1 M $CuSO_4$ concentration in Cu-presence and Cu-absence

In presence of Cu, the hydrogels degraded with a rate inversely proportional to the gelatin concentration. When Cu was removed, similar trend was observed for all the hydrogels though each of them swelled initially.

4.8. HEMOCOMPATIBILITY

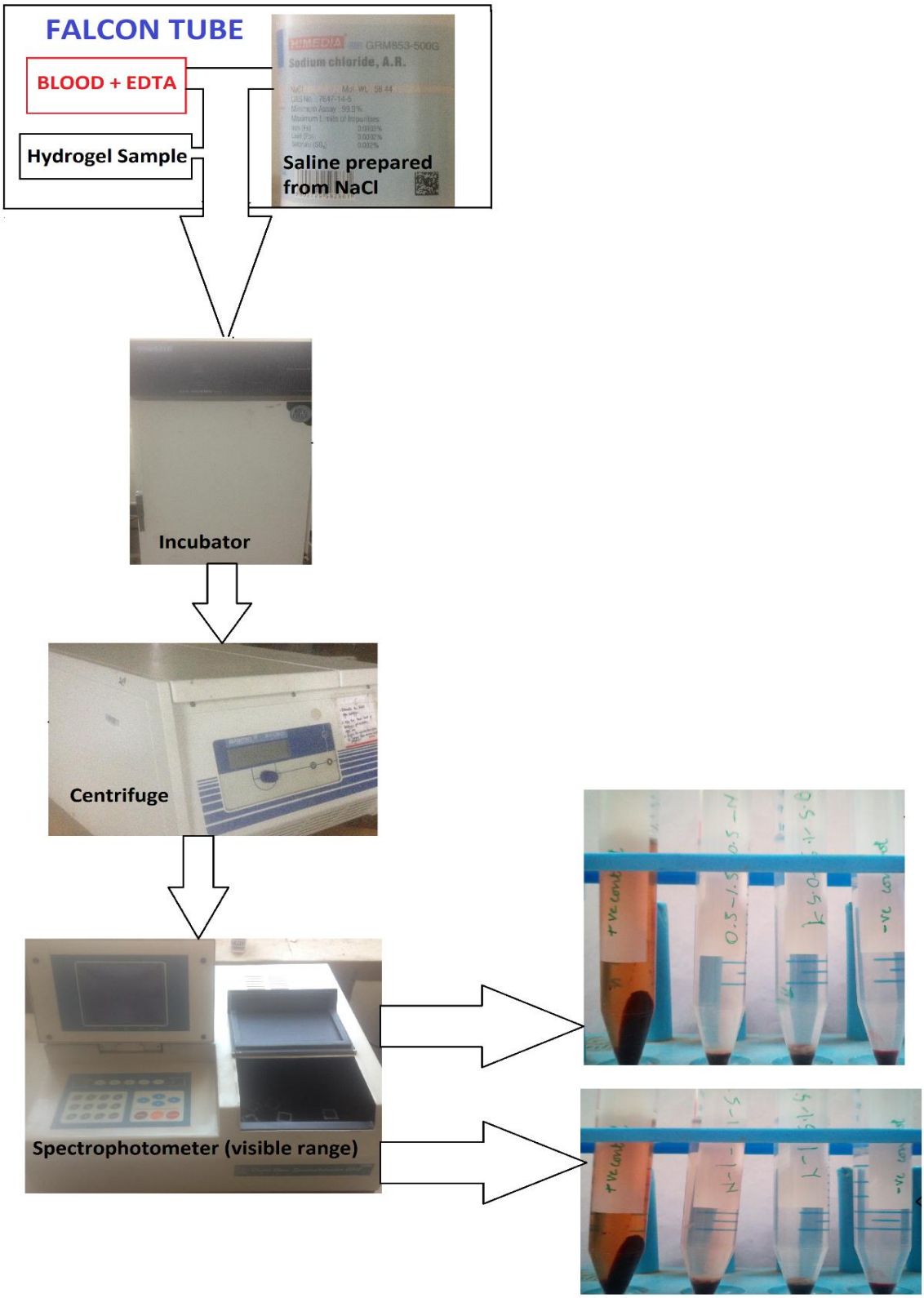


Fig.4.17: Complete procedure of Hemocompatibility along with the output

Sample	**O.D. value**	**Hemolysis %**
+ve control	*0.324*	*100*
-ve control	*0.104*	*0*
0.5-1.5-0.5-y	0.108	1.8182
0.5-1.5-0.5-n	0.104	0
0.5-1.5-1-y	0.105	0.4545
0.5-1.5-1-n	0.114	4.5455

Table.4.3: Hemolysis % of the samples

According to ASTM standards, in Hemocompatibility test, if the biomaterial has % hemolysis less than 10, it is found to be hemocompatible and if it is greater than 10 then the biomaterial is not compatible in nature. If the % hemolysis is less than 5, the biomaterial is said to be highly hemocompatible. The hemolysis % of all the measured samples were found to be below 5%. Hence all the samples are hemocompatible and hence biocompatible irrespective of presence of Cu and absence of Cu. From this result, we could find that the Cu presence in the samples is not of the toxic level if all the excess Cu ions are removed away from the samples.

CHAPTER 5

CONCLUSION

5. CONCLUSION

According to Saraai et. al [18], various concentrations of gelatin and sodium alginate can be used to develop micro beads using ionotropic gelation with proper balance between mechanical strength and swelling property. In this research, ionotropic gelation was employed to develop the uniform and uniaxial porous structures of hydrogel containing gelatin and alginate to achieve the said balance. The physico-chemical and structural characterization of the hydrogels showed various enhanced properties in comparison to the individual properties of either gelatin or alginate.

REFERENCE

1. Kalfas IH. *Principles of Bone Healing*, Neurosurgical Focus 2001, 1-4.
2. Oryan A, Alidadi S, Moshiri A, Maffulli N. *Bone regenerative medicine: classic options, novel strategies, and future directions*, Journal of Orthopaedic Surgery and Research 2014, 9-18.
3. Marshall A. *Tissue Engineering*, Nature Biotechnology 2000, 239-356.
4. Williams D. *Benefit and risk in tissue engineering*, Materials Today 2004, 24-29.
5. Carletti E, Motta A, Migliaresi C. *Scaffolds for tissue engineering and 3D cell culture*, Methods of Molecular Biology 2011, 17-39.
6. Chan BP, Leong KW. *Scaffolding in tissue engineering: general approaches and tissue-specific considerations*, European Spine Journal 2008, 467–479.
7. Discher DE, Janmey P, Wang YL. *Tissue cells feel and respond to the stiffness of their substrate*, Science 2005, 1139–1143.
8. Kopecek J. *Hydrogel biomaterials: a smart future*, Biomaterials 2007, 5185–5192.
9. Lutolf MP. *Biomaterials: Spotlight on hydrogels*, Nature Materials 2009, 451–453.
10. Chung HK, Park TG. *Self-assembled and nanostructured hydrogels for drug delivery and tissue engineering*, Nano Today 2009, 429–437.
11. Oh JK. *Engineering of nanometer-sized cross-linked hydrogels for biomedical applications*, Canadian Journal of Chemistry 2010, 173–184.
12. Ulijn RV, Bibi N, Jayawarna V. *Bioresponsive hydrogels*, Materials Today 2007, 40–48.
13. Lee J, Cuddihy MJ, Kotov NA. *Three-dimensional cell culture matrices: state of the art*, Tissue Engineering Part B 2008, 61–86.

14. Zhu J. *Bioactive modification of poly (ethylene glycol) hydrogels for tissue engineering*, Biomaterials 2010, 4639–4656.
15. Geckil H, Xu F, Zhang XH, Moon S, Demirici U. *Engineering hydrogels as extracellular matrix mimics*, Nanomedicine 2010, 469–484.
16. Hunt NC, Grover LM. *Cell encapsulation using biopolymer gels for regenerative medicine*, Biotechnology Letters 2010, 733–742.
17. Drury JL, Mooney DJ. *Hydrogels for tissue engineering: scaffold design variables and applications*, Biomaterials 2003, 4337–4351.
18. Saarai A, Kasparkova V, Sedlacek T, Saha P. *On the development and characterisation of crosslinked sodium alginate/gelatine hydrogels*, Journal of the Mechanical Behavior of Biomedical Materials 2013, 152-166
19. Ehrler DM, Vaccaro AR. *The use of allograft bone in lumbar spine surgery*, Clinical Orthopaedics and Related Research 2000, 38–45.
20. Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. *Hydrogels in regenerative medicine*, Advanced Materials 2009, 3307–3329.
21. Nicodemus GD, Bryant SJ. *Cell encapsulation in biodegradable hydrogels for tissue engineering applications*, Tissue Engineering Part B: Reviews 2008, 149–165.
22. Cushing MC, Anseth KS. *Hydrogel cell culture*, Science. 2007, 1133–1134.
23. Nuttelman CR, Rice MA, Rydholm AE, Salinas CN, Shah DN, Anseth KS. *Macromolecular monomers for the synthesis of hydrogel niches and their application in cell encapsulation and tissue engineering*, Progress in Polymer Science 2008, 167-173
24. Brandl F, Sommer F, Goepferich A. *Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behaviour*, Biomaterials 2007, 134–146.

25. Varghese S, Elisseeff JH. *Hydrogels for musculoskeletal tissue engineering*, Advances in Polymer Science 2006, 95–144.
26. Bode F, da Silva MA, Drake AF, Ross-Murphy SB, Dreiss CA. *Enzymatically cross-linked tilapia gelatin hydrogels: Physical, chemical, and hybrid networks*, Biomacromolecules 2011, 3741–3752.
27. Dash R, Foston M, Ragauskas JA. *Improving the mechanical and thermal properties of gelatin hydrogels cross-linked by cellulose nanowhiskers*, Carbohydrate Polymers 2013, 638–645.
28. Smidsrod O, Skjak-Bræk G. *Alginate as immobilization matrix for cells*, Trends in Biotechnology 1990, 71–78.
29. Rinaudo M. *Main properties and current applications of some polysaccharides as biomaterials*, Polymer International 2008, 397–430.
30. Clark DE, Green HC. *Alginic acid and process of making same*, US Patent 2036922, 1936.
31. Park K, Sharaby WSW, Park H. *Physical Gels, Biodegradable Hydrogels for Drug Delivery*, Technomic Publishing 1993, 99–140.
32. Gulrez SKH, Al-Assaf S, O Phillips G. *Hydrogels: Methods of Preparation, Characterisation and Applications*, Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications 2011, 92-111
33. Rinaudo M. *Main properties and current applications of some polysaccharides as biomaterials*, Polymer International 2008, 397–430.
34. Augst AD, Kong HJ, Mooney DJ. *Alginate hydrogels as biomaterials*, Macromolecular Bioscience 2006, 623–633.

35. Despang F, Dittrich R, Gelinsky M. *Novel Biomaterials with Parallel Aligned Pore Channels by Directed Ionotropic Gelation of Alginate: Mimicking the Anisotropic Structure of Bone Tissue*, Advances in Biomimetics, 349-374.
36. Saravu K, Jose J, Bhat MN, Jimmy B, Shastry B. *Acute ingestion of copper sulphate: A review on its clinical manifestations and management*, Indian Journal of Critical Care Medicine 2007, 74–80.
37. Bajpai SK, Sharma S. *Investigation of swelling/degradation behaviour of alginate beads crosslinked with Ca2þ and Ba2þ ions*, Reactive & Functional Polymers 2004, 129–140.
38. Amarnath LP, Srinivas A, Ramamurthi A. *In vitro hemocompatibility testing of UV-modified hyaluronan hydrogels,* Biomaterials 2006, 1416-1424.
39. Satapathy S. *Development of gelatin based hydrogel, emulsion hydrogel, bigel :- a comparative study*, MTech thesis 2014
40. Read D, Smith D, Taber KS, Crabb E, Fleming D. *Dissolving copper in Nitric Acid*, Education in Chemistry 2011, 134-157